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INDICE

Welcome address of prof. Franco Scaramuzzi

Dear Colleagues, Ladies and Gentlemen,
on Georgofili's behalf I am honoured to welcome all of you in our Accademia for a Meeting on "Molecular aspects of olfaction and applications in agriculture". The discovery of olfactory receptors provided in 1991 by biochemical studies, opened interesting perspectives in basic and applied science, pointing out the peculiar regenerative capacity of olfactory neurones and the plasticity of central nervous system. The possibility to define a code of odours, the use of pheromones in the control of insects populations and the improved evaluation of the organoleptic properties of foods are some of the more interesting applications of the new scientific knowledge.

The Georgofili are very honoured to host today personally prof. Linda Buck, Nobel Price for Medicine and Physiology 2004. At the same time, I am glad to welcome and express our thanks to the other Speakers: the Scientists Paolo Pelosi, Walter Leal, Aldo Fasolo and Krishna Persaud, that have kindly accepted our invitation. I want to express our gratitude also to the Foundation Cassa di Risparmio di Lucca for the financial support enabling this Meeting.

I would now ask prof. Pelosi to take the floor and the Chairmanship of the meeting.

Thank you

Ringraziamenti

L'Accademia dei Georgofili desidera esprimere il più vivo ringraziamento ai relatori che hanno reso possibile questo incontro, in particolare al premio Nobel Linda Buck.
Un ringraziamento particolare per la Fondazione della Cassa di Risparmio di Lucca, che ha generosamente contribuito alla organizzazione del Convegno e alla pubblicazione di questo supplemento agli Atti dell'Accademia.

Acknowlegements

The "Accademia dei Georgofili" thanks the speakers, in particular the Nobel laureate Linda Buck, for making this Meeting feasible.
Special thanks to the "Fondazione della Cassa di Risparmio di Lucca" for their generous contribution to the organization of the Conference and the publication of these Proceedings.

Introduction

In the last two decades the study of olfaction has experienced an explosive growth and stimulated wide interest in many fields of scientific research. Until comparatively recently, the attention of scientists was only focused on odorous molecules, without any attempt to decipher how the messages encoded in molecular structures could elicit sensations, emotions, or behavioural reactions. Olfaction, therefore, was a territory of chemists, in particular those interested in perfumery. Because of the commercial aspects of processed foods, and increased consumer awareness of the concept of quality, a second important field attracted the interest of scientists, that of food flavours and the organoleptic properties of foods. Odours have been identified as one of the major elements of food quality. More recently, the increasing pollution of air, water and soil, together with a greater awareness of the impact of such problems, stimulated scientific research on environmental odours.

Several aspects of our life are greatly affected by our olfactory experience, but in humans the perception of odours mainly influences hedonic aspects, without being essential for survival.

On the other hand, most animal species rely on the correct functioning of their olfactory system for survival of the individual and for the conservation of the species. Sex pheromones finely regulate interactions between sexes, avoiding interspecies mating that would result in infertility. Olfaction has the essential functions of selecting good food and for detecting and tracking prey or predators. Finally in social species, odours indicate ranks and roles and regulate the complex relationships between individuals both within a colony and between different colonies.

How animals perceive and recognise a great number of different odours remained a mystery long after the fine art of perfumery was well established

and chemists were able to synthesize natural odours. The biochemical mechanisms of odour perceptions and the proteins involved in olfactory transduction were completely unknown at the end of the 1970s, when even the existence of receptor proteins for odours was a matter of debate.

In the long search for olfactory receptors, soluble proteins capable of binding odorant molecules (OBPs: odorant-binding proteins) were discovered at the beginning of the 1980s, but it was only in 1991 that the genes encoding olfactory receptors were finally identified by Linda Buck and Richard Axel. Their paper represents a milestone in the history of olfaction and immediately stimulated an explosive interest in this field that had remained neglected for such long time. The impact of the discovery also provided the necessary tools for marking the lines connecting the neurons of the olfactory mucosa to the olfactory bulbs and understanding how the messages encoded in the molecular structures of the odorants are translated into electric signals and later amplified and processed to produce sensations, emotions, behavioural responses. Linda Buck and Richard Axel provided a major contribution to understanding such aspects, a work that, together with the discovery of olfactory receptors won them the Nobel Prize for Medicine or Physiology in 2004.

Linda Buck continued her research, following the olfactory signals from the bulbs into the brain cortex, discovering an unexpectedly complex pattern of interactions between the neurons in such area. These most recent studies, that are the topic of her presentation, show how olfactory signals are processed and modified as they travel from the periphery to the central areas of the brain. The most dramatic effects are evident with mixtures of odorants that convey unique images to the brain, deriving not just from the superimposition of individual signals as produced by the single components, but from the various and unpredictable interactions of such signals. The practical implications of such discoveries are evident if we only consider that food aroma is almost always due to a great number of chemical components.

The lecture of Linda Buck is followed by four presentations on other topics of olfactory research. The first of these presents the structures and the properties of odorant-binding proteins. The physiological role of such soluble proteins, that represented the first biochemical elements of the olfactory system to be discovered, is still unclear, despite the great amount of structural information available for many members of this class, both in vertebrates and in insects.

The second contribution deals with another interesting aspect of the olfactory neurons, their unique property of regenerating. The presence of stem

cells at a very early stage make the olfactory system extremely plastic and an ideal tissue for studying development and regenerations of nerve cells.

The third lecture presents some aspects of insect pheromone perception, describing the chemical structure of pheromones, their interactions with binding proteins and their behavioural effects. The object of this study is the navel orangeworm, one of the major pests in California, producing massive damage to several fruits.

The final talk examines the possibility of measuring odours, taking as a model the physiological olfactory system and describes the available technology and the current sensors for an "artificial nose". Although such instruments look very primitive, when compared with the high complexity of the physiological olfactory system, they have been successfully applied in monitoring environmental odours or evaluating the organoleptic quality of some foods.

The understanding of olfactory perception and recognition at the molecular level, besides representing one of the major scientific discoveries of last century, is the basis for several applcations of great economic interest. The fields of agriculture and food science in particular already benefit from the wide information made available. Two well known examples are the use of pheromones in the control of insect populations and the evaluation of the aroma of foods as a major parameter for assessing their quality. The basic research in olfaction has suggested new techniques and strategies and at the same time has provided the background information and model for the construction of "artificial noses".

The opening of integrated multidisciplinary research in all aspects of olfaction and chemoreception will have a large impact in our lives for the future.

Carlo Galoppini and Paolo Pelosi

Linda B. Buck*

Unraveling the Sense of Smell

INTRODUCTION

Humans and other mammals can perceive a vast number and variety of chemicals in the external world. The sense of smell is governed by the olfactory system, a system characterized by exquisite sensitivity and discriminatory power (Kandel et al., 2000). It is speculated that humans can sense as many as 10,000 to 100,000 chemicals as having a distinct odor. All of these odorants are small, volatile molecules. However, they have diverse structures, and somehow those different structures are perceived as having different odors. Even odorants with nearly identical structures can have odors as different as sweaty and orange.

In addition to odorants, the olfactory system detects pheromones, chemicals released from animals that act on members of the same species, stimulating hormonal changes or instinctive behaviors, such as aggression or mating (Kandel et al., 2000). It also detects predator odors that can elicit an innate fear response in animals.

Here, I will discuss two questions that have been of interest to us. First, how does the olfactory system detect and distinguish so many different chemicals? And second, how does the brain translate those chemicals into diverse perceptions and behaviors?

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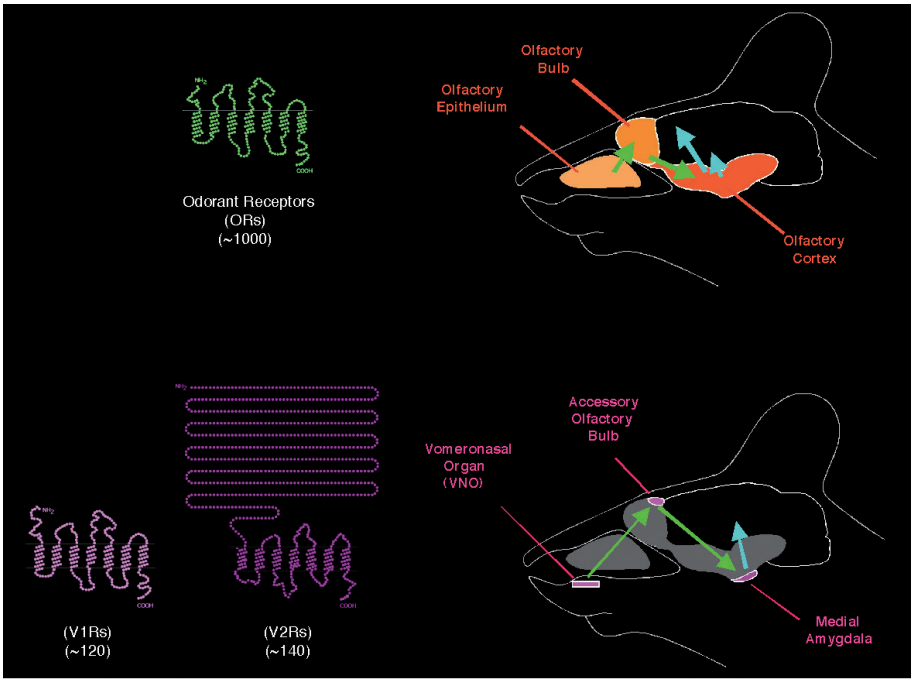

Fig. 1 *Two olfactory pathways. While odorants are detected in the olfactory epithelium, many pheromones are detected in the VNO. From those two sites, sensory signals travel through different pathways in the brain. The olfactory epithelium expresses a large family of odorant receptors whereas the VNO expresses two smaller families of chemosensory receptors*

TWO OLFACTORY PATHWAYS

Odorants are first detected by olfactory sensory neurons in the olfactory epithelium that lines the nasal cavity (fig. 1) (Kandel et al., 2000). These neurons transmit signals to the olfactory bulb of the brain, which in turn relays signals to the olfactory cortex. From there, olfactory information is sent to other brain areas. These include higher cortical areas thought to be involved in odor discrimination and deep limbic areas of the brain, which are thought to mediate the emotional and physiological effects of odors.

In contrast, pheromones are thought to be detected primarily in the vomeronasal organ, or VNO, a separate olfactory structure in the nasal septum (fig. 1) (Wysocki and Lepri, 1991; Meredith, 1998; Halpern and Martinez-Marcos, 2003). Signals from VNO neurons are relayed through the accessory olfactory bulb to the medial amygdala and then the hypothalamus, an area

that controls hormone levels and a variety of instinctive behaviors. Although most mammals, and some lower vertebrates, have a VNO, this structure is lacking in humans.

RECEPTORS FOR ODORANTS AND PHEROMONES

Over the past fifteen years, we and others have identified three different families of chemosensory receptors in the olfactory system: one large family of odorant receptors (ORs) in the olfactory epithelium of the nose, which Richard Axel and I identified in 1991 (Buck and Axel, 1991), and two smaller families of receptors in the VNO that we and others found in later studies (fig. 1) (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997).

All three types of olfactory receptor have the seven transmembrane domain structure characteristic of G protein-coupled receptors as well as key peptide motifs typical of such receptors. Members of each olfactory receptor family vary extensively in protein sequence, suggesting that each family is likely to recognize a variety of different chemicals.

Analyses of human and mouse genome sequences by our lab and others indicate that humans have about 350 different ORs (Glusman et al., 2001; Zozulya et al., 2001; Malnic et al., 2004) and mice have ~1000 (Young and Trask, 2002; Zhang and Firestein, 2002; Godfrey et al., 2004). In both species, OR genes are highly distributed in the genome and are found on most chromosomes. For example, in the mouse, we identified OR genes at 51 distinct loci on 17 different chromosomes (Godfrey et al., 2004).

ODOR CODING IN THE OLFACTORY EPITHELIUM

How does the olfactory system organize signals from 1000 different ORs? Early studies in our lab and others identified four spatial zones in the olfactory epithelium that express different sets of OR genes (fig. 2A) (Ressler et al., 1993; Vassar et al., 1993). Each OR gene is expressed in about 1/1000 neurons and those neurons are randomly scattered within one zone.

These findings indicated, first, that input from one type of OR is highly distributed in the epithelium. Thus neurons with receptors for different odorants must be interspersed. They further indicated that each neuron might

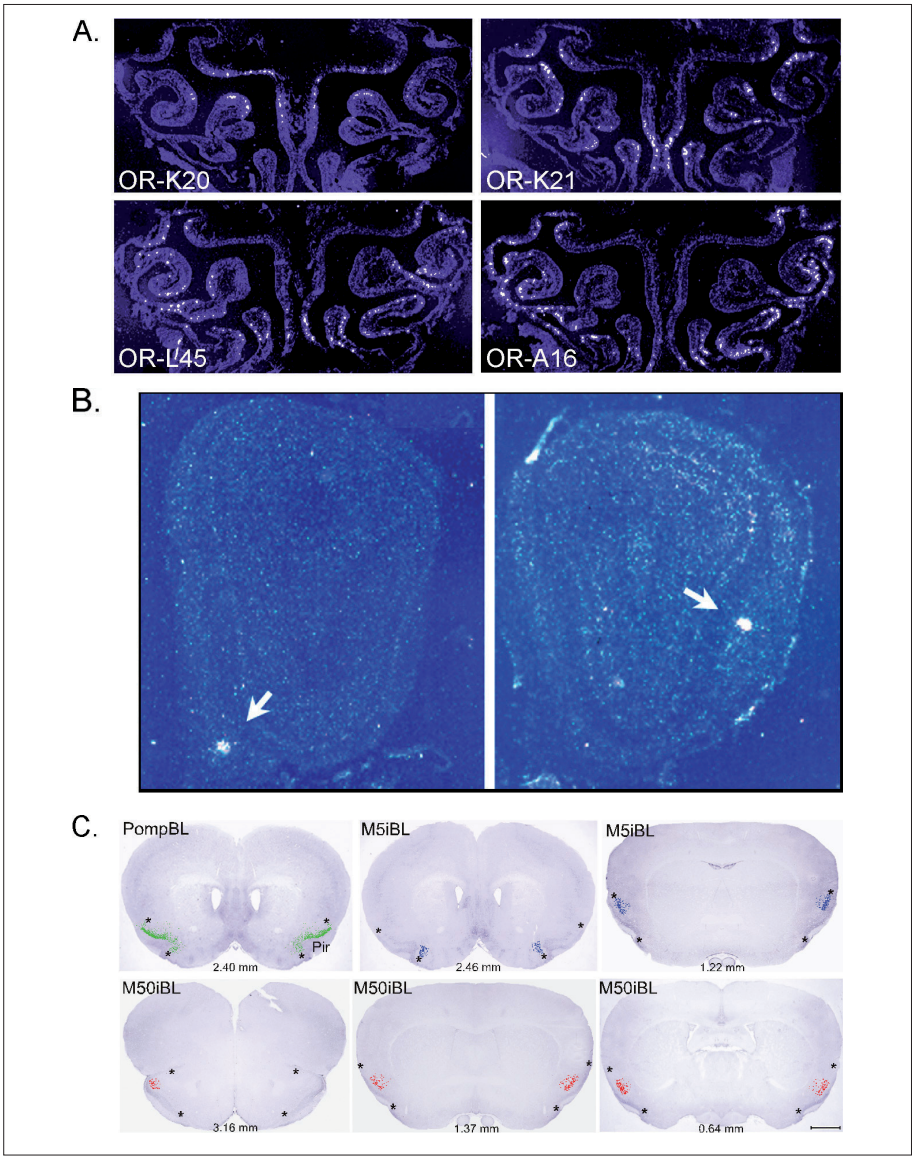

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Fig. 2 *The organization of odorant receptor signals in the olfactory pathway. Shown here are the patterns of hybridization seen with four different OR gene probes in the olfactory epithelium (A), the hybridization pattern seen with one OR probe in the olfactory bulb (B), and the locations of BL+ neurons in the anterior piriform cortex of mice coexpressing BL in all olfactory sensory neurons (OMP-BL) or with a single OR gene (M5iBL or M50iBL) (C). A schematic showing OR inputs at these three locations is shown in D*

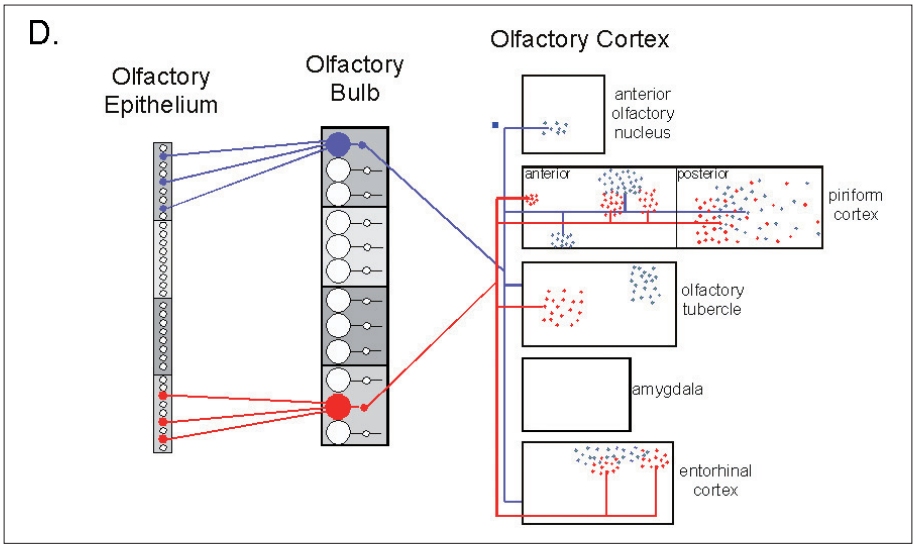

Fig. 2

express only one OR gene. We later confirmed this by examining gene expression in single olfactory sensory neurons using RT-PCR (reverse transcriptase-polymerase chain reaction) (Malnic et al., 1999). Thus, in the nose, inputs from different ORs are segregated in different neurons, and the information that each neuron transmits to the brain is derived from a single receptor type.

The VNO has a similar organization, but, here, V1Rs and V2Rs are expressed in different zones and the different zones also have different G proteins (Dulac and Axel, 1995; Halpern et al., 1995; Berghard and Buck, 1996; Herrada and Dulac, 1997; Matsunami and Buck, 1997). Each receptor gene is expressed in a small percentage of neurons that are scattered throughout one zone, suggesting that, as in the nose, each neuron may transmit signals to the brain that are derived from a single receptor type.

COMBINATORIAL RECEPTOR CODES FOR ODORS

How does the OR family encode the unique identities of a vast number of odorants? To address this question, we searched for ORs that recognize specific odorants. We first used calcium imaging to identify single mouse olfactory sensory neurons that respond to specific odorants. We then used single cell

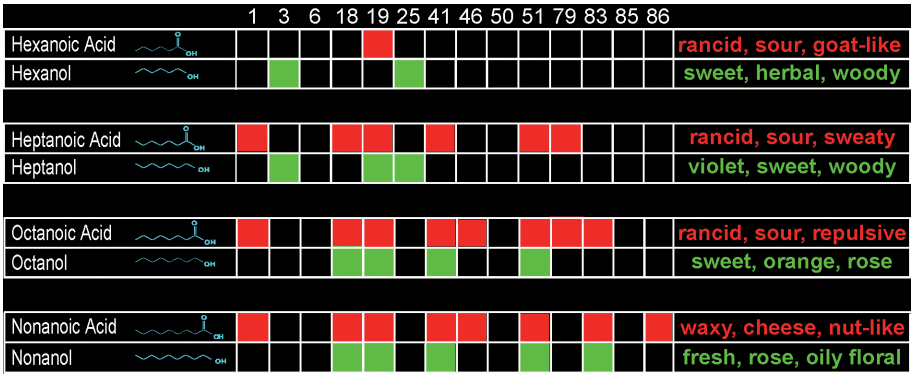

Fig. 3 *Combinatorial receptor codes for odors. Different odorants are detected by different combinations of ORs, but each OR can detect multiple odorants. As shown here, odorants differing by a single functional group have different receptor codes, explaining how they elicit different odor perceptions*

RT-PCR to identify the OR gene expressed by each responsive neuron (Malnic et al., 1999).

These experiments showed that one odorant is recognized by multiple ORs and each OR can recognize multiple odorants, but, importantly, different odorants are detected by different combinations of ORs (fig. 3). This indicated that the OR family is used in a combinatorial fashion to encode odor identities. Different odorants are detected, and thereby encoded, by different combinations of ORs, but each OR serves as one component of the codes for many odorants. Different odorants have different receptor codes. Even if each odorant were detected by only 3 ORs, this combinatorial scheme could potentially generate almost one billion different odor codes.

These studies also provided potential explanations for several puzzling features of human odor perception. Changing the structure of an odorant even slightly can alter its perceived odor. The aliphatic acids and alcohols we used in our studies are excellent examples of this phenomenon (fig. 3). The acids all have unpleasant odors, such as rancid, sour, or sweaty. In contrast, aliphatic alcohols have pleasant odors, such as herbal, woody, or orange. We found that acids and alcohols that differed by a single functional group were invariably recognized by different combinations of ORs (fig. 3). Thus a slight change in the structure of an odorant can alter its receptor code and thereby change its perceived odor.

We found that changing the concentration of an odorant also changes its receptor code. At higher concentrations, additional ORs were invariably

recruited into the odor response. This may explain why it is that changing the concentration of an odorant can also change its perceived odor.

ODOR CODING IN THE OLFACTORY BULB

Olfactory sensory neurons in the nose transmit signals to mitral cell relay neurons in the olfactory bulb. In the bulb, the axons of sensory neurons synapse with the dendrites of mitral cells in about 2000 spherical structures, called glomeruli. Each sensory neuron and each mitral cell synapses in a single glomerulus.

In the olfactory bulb, we and others found an arrangement very different from what we had found in the olfactory epithelium (Ressler et al., 1994; Vassar et al., 1994). Here, single OR probes labeled OR mRNA in sensory axons in only one to two glomeruli located at two spots, one on the medial side of the bulb and one on the lateral side (fig. 2B). Different OR probes labeled different glomeruli. And surprisingly, those glomeruli had nearly identical locations in different individuals.

These results indicated that sensory information that is broadly organized in the nose is transformed in the bulb into a stereotyped sensory map (fig. 2D). In this map, inputs from different ORs are targeted to different glomeruli and the bulb neurons associated with those glomeruli. The map is virtually identical in different individuals.

The olfactory epithelium and bulb have one important thing in common, however. At both sites, signals from different ORs are segregated. Each sensory neuron in the epithelium and each glomerulus and relay neuron in the bulb appears to be dedicated to only one type of OR.

The structure of the bulb map is likely to be important in at least two respects. First, it is likely to maximize sensitivity to low concentrations of odorants. Signals from 5000 or so sensory neurons with the same OR converge on two to four glomeruli and about 50 mitral cells, allowing a high degree of signal integration. Second, the bulb map is likely to be important for the stimulation of odor memories. Olfactory sensory neurons are short-lived cells that are continuously replaced from an underlying layer of stem cells in the olfactory epithelium. However, the bulb map remains constant over time. As a result, the neural code for an odor remains intact, assuring that odorants can elicit distant memories.

Given the combinatorial use of ORs in odor coding, the organization of OR inputs in the olfactory epithelium and bulb indicated that the code for an

odorant in the nose is a dispersed ensemble of sensory neurons, each expressing one OR component of the odorant's receptor code. In contrast, the code for an odorant in the bulb is a specific combination of glomeruli that receive inputs from those ORs and have the same spatial arrangement in different individuals. Studies of odor-induced neural activity are consistent with this arrangement.

ODOR CODING IN THE OLFACTORY CORTEX

What happens to the combinatorial OR inputs at higher levels of the nervous system to ultimately generate different odor perceptions? Mitral cell relay neurons in the olfactory bulb extend axons to the olfactory cortex, a large area that stretches along the ventral lateral part of the brain (fig. 1) (Kandel et al., 2000). The olfactory cortex is composed of a number of distinct anatomical areas, at least some of which are likely to have different functions. The two largest areas are the anterior and posterior piriform cortices, which are also referred to as "primary olfactory cortex".

We were initially interested in three questions regarding the olfactory cortex ("cortex"). First, do different areas of the cortex, which may have different functions, receive signals derived from different subsets of ORs or does each area receive input from the entire OR repertoire? Second, is input from one OR scattered in the cortex, as in the nose, targeted to unique, stereotyped sites, as in the bulb, or organized in some other way? And finally, given that each odorant is recognized by multiple ORs, are signals from different ORs combined in individual cortical neurons, or are they segregated in different neurons as in the nose and bulb?

Before we could address these questions, we first had to develop a method that would allow us to analyze OR inputs in the cortex. In initial experiments, we asked whether it would be possible to trace neural circuits genetically. In transgenic OMP-BL mice that expressed barley lectin, or BL, in all olfactory sensory neurons, we found that BL crossed two synapses to label downstream neurons in both the bulb and cortex (Horowitz et al., 1999).

We next used gene targeting to make "knockin mice" that coexpressed BL with only one OR gene, the M5 OR gene or the M50 OR gene (Zou et al., 2001). We did this by inserting an IRES sequence followed by a BL gene just 3' to the OR coding region. The IRES sequence allows the OR and BL proteins to be independently translated from the same mRNA. In the knockin mice, BL was produced only in neurons that expressed the M5 or M50 OR.

In the olfactory cortex, the axons of bulb neurons branch and form synapses in Layer I with the dendrites of pyramidal neurons whose cell bodies are located in Layers II and III (Neville and Haberly, 2004). In OMP-BL mice, which express BL in all olfactory sensory neurons, we saw labeled neurons in Layers II and III throughout the olfactory cortex (Zou et al., 2001).

In contrast, in the M5 and M50 knockin mice, BL-labeled neurons were found only in distinct clusters (fig. 2C) (Zou et al., 2001). In each knockin strain we detected two to three loose clusters of BL+ neurons in the anterior piriform cortex and individual clusters in several other areas of the olfactory cortex. Most clusters were bilaterally symmetrical in the left and right brain. The density of BL+ neurons was highest in the center of each cluster, but, even there, only about 50% of neurons were BL+. Detailed analysis of the labeled clusters in the anterior piriform cortex showed that they had similar locations and dimensions in different individuals. The M5 and M50 clusters had different locations, but one M5 cluster appeared to overlap partially with one M50 cluster.

These results indicated that the olfactory cortex has a stereotyped map of OR inputs (fig. 2D). In this map, signals derived from one type of OR are targeted to five to six loose clusters of cortical neurons whose locations are virtually identical among individuals.

These findings clearly indicate that input from one OR diverges to multiple areas of the olfactory cortex. This may allow a parallel processing of OR inputs in which inputs from the same ORs are combined or modulated in different ways prior to transmission to other brain regions that have different functions.

It also appears, however, that at least one cortical area may receive input from only a subset of ORs. In both knockin strains, the olfactory amygdala was devoid of BL+ neurons even though this area contained many BL+ neurons in OMP-BL mice (fig. 2D). Interesting, the olfactory amygdala transmits signals to parts of the hypothalamus that do not receive inputs from any other area of the olfactory cortex. Given that the hypothalamus controls a variety of basic drives and instinctive behaviors, it may be that the olfactory amygdala receives input only from ORs relevant to those functions.

In one major olfactory cortical area, the anterior piriform cortex, about 3% of pyramidal neurons were BL+ and the clusters containing those neurons occupied about 5% of the total area. This indicated that the OR input map in the cortex is dramatically different from that in the bulb. First, while signals from different ORs are spatially segregated in different glomeruli in the bulb, they are likely to overlap extensively in the cortex. Second, while

each neuron in the nose and bulb is dedicated to one OR, each cortical neuron is likely to receive combinatorial inputs from multiple different ORs. This arrangement could conceivably allow single cortical neurons to integrate signals from different ORs that recognize the same odorant.

ODOR REPRESENTATIONS IN THE CORTEX

The genetic tracing studies discussed above showed how OR inputs are organized in the olfactory cortex. However, because of the complexity of OR inputs in the cortex and the combinatorial use of ORs in odor coding, it was impossible to predict how odorants are actually represented in the cortex. To address this question, we exposed mice to a number of different odorants and then examined odor response patterns in the anterior piriform cortex(Zou et al., 2005). To do this, we used immunostaining of brain sections to look at the induction of c-Fos, a marker of neuronal activity.

These studies showed that that one small cortical region can respond to more than one odorant, but that different locations respond to different sets of odorants. To visualize cortical patterns of neuronal activation, we constructed diagrams showing the positions of c-Fos+ neurons across the entire piriform cortex of mice exposed to different odorants.

Our results indicated that each odorant activates a small subset of cortical neurons that are sparsely distributed over a relatively large area. Different odorants elicited different patterns of cortical activation. However, the activation patterns for different odorants partially overlapped. These results indicated that, first, odor representations in the cortex are highly distributed and, second, the representations of different odorants are mulitplexed in partially overlapping neuronal arrays.

The odorant response patterns in the cortex were similar, though not identical, in different individuals. Comparisons of different individuals identified hot spots for odorants, cortical regions that were usually or always activated by a given odorant. Neurons activated by individual odorants were distributed over a larger area than were inputs from either the M5 or M50 OR visualized by genetic tracing. This indicated that signals from different ORs that recognize the same odorant can be targeted to different locations in the anterior piriform cortex, though an indeterminate number could be targeted to the same, or partially overlapping, locations.

Interestingly, we found that related odorants stimulated similar patterns, even though two of the odorants shared only a single functional group. One

possible explanation is that the related odorants are detected by many of the same ORs. Another possibility that we are currently exploring is that there is a molecular logic underlying the organization of OR inputs in the cortex. This logic could be related to odorant structure, OR structure, or both.

CORTICAL NEURONS AS COINCIDENCE DETECTORS

Using low concentrations of odorants to mimic the natural environment, single odorants induced c-Fos expression in about 500-800 neurons in the anterior piriform cortex, a result consistent with previous electrophysiological studies. In contrast, the genetic tracing studies indicated that input from one OR is targeted to about 5000 neurons in this cortical area. Furthermore, each odorant is recognized by a combination of different ORs.

What is the explanation for this numerical difference in OR inputs versus odor responses? Given that each cortical neuron appears to receive input from multiple different ORs, one intriguing possibility was that the cortical neurons function as coincidence detectors. In this model, activation of a cortical neuron would require simultaneous inputs derived from more than one type of OR. If this model is correct, a binary mix of odorants should stimulate neurons beyond those stimulated by its single components because merging the receptor codes of the two odorants would create new combinations of OR inputs.

To test this idea, we used fluorescence *in situ* hybridization to look at odor-induced expression of mRNA encoded by the immediate gene, *Arc* (Zou and Buck, 2006). Initial experiments showed that exposure of mice to odorants induces the expression of *Arc* mRNA in a subset of pyramidal neurons in the anterior piriform cortex. Similar to what was previously reported for depolarized hippocampal neurons, *Arc* mRNA was localized to one to two bright spots in the nucleus of these neurons five minutes following odorant exposure, but then traveled to the cytoplasm, where it was found exclusively in most *Arc*+ neurons after thirty minutes.

We next exposed mice to the odorant eugenol, waited either five minutes or thirty minutes, and then examined *Arc* staining patterns in neurons across the entire anterior piriform cortex. After five minutes, most labeled neurons had *Arc* only in the nucleus, but after thirty minutes most had *Arc* only in the cytoplasm.

We then compared the responses of cortical neurons to binary odorant mixes versus their individual components (fig. 4A). We did this for three different

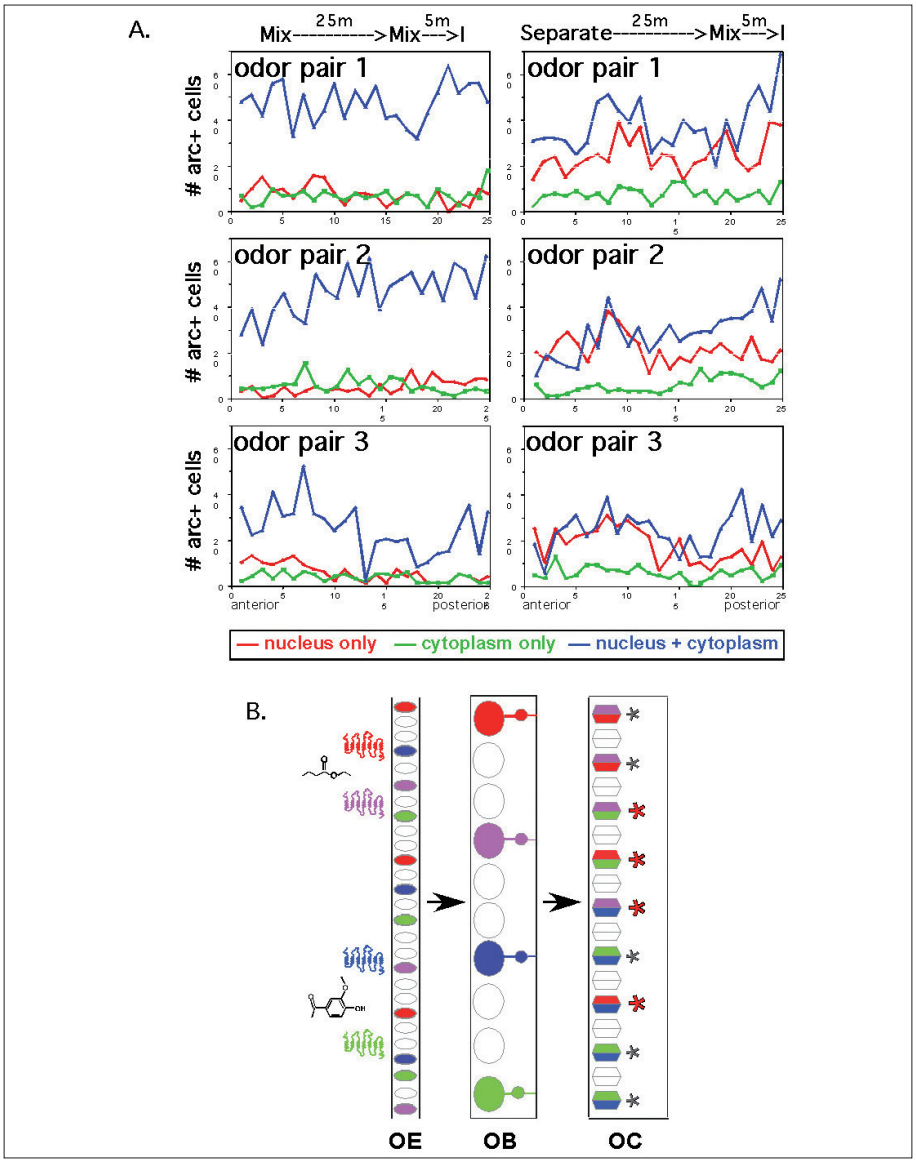

Fig. 4 *Coincidence detection in the olfactory cortex. Shown here are the numbers of neurons with Arc mRNA in different subcellular patterns in sections spanning the anterior-posterior axis of the anterior piriform cortex following exposure of mice to binary odorant mixes (A). With two exposures to the same mix (left), most neurons had Arc in both nucleus and cytoplasm because they responded to the mix both times. However, when two odorants were first given separately and then as a mix (right), many neurons had only nuclear Arc, indicating that they responded to the mix, but not either odorant alone. These results are consistent with a model in which olfactory cortical neurons require simultaneous inputs from different ORs for their activation (B)*

pairs of odorants. When mice were exposed to an odor mix twice, at both minus thirty and minus five minutes, most labeled neurons had *Arc* signal in both the nucleus and cytoplasm. These neurons responded to both exposures to the mix. In contrast, when animals were exposed to two odorants first separately and then as a mix, many neurons had *Arc* only in the nucleus. These neurons were activated by the mix, but not by either of the single odorants in the mix.

When an odorant mix was given twice, only 7-10% of neurons responded to the second exposure alone. In contrast, when two odorants were first given separately and then as a mix, 36-39% of neurons responded only to the second exposure. These neurons responded to the mix, but not to either of its single components.

These findings are consistent with the idea that neurons in the olfactory cortex act as coincidence detectors whose activation requires simultaneous input from more than one OR. In the simple version of this model, two odorants are each recognized by two ORs. Inputs from those ORs are segregated in both the olfactory epithelium and olfactory bulb, but are combined in some cortical neurons, which are thereby activated (fig. 4B). When the two odorants are combined, additional cortical neurons are activated due to the new combinations of OR inputs. The stimulation of additional neurons could also involve excitatory connections among the cortical neurons.

These results contrast sharply with studies of the bulb showing that bulb relay neurons that respond to a binary odorant mix also generally respond to one component of the mix (Giraudet et al., 2002; Lin et al., 2005). The olfactory cortex thus appears to have a synthetic ability that is lacking in the olfactory bulb. It is possible that the results we obtained represent a first step in the reconstruction of an odor image from its deconstructed features, which are carried by combinations of OR inputs. Interestingly, these studies may also explain why mixing different odorants together can create novel odor perceptions in humans.

NEURAL CIRCUITS THAT MEDIATE PHEROMONE EFFECTS ON REPRODUCTION

In other studies, we have begun to explore how pheromones elicit hormonal changes and instinctive behaviors. In mice, pheromones can stimulate sexual behaviors as well as changes in reproductive hormones (Wysocki and Lepri, 1991; Meredith, 1998; Halpern and Martinez-Marcos, 2003).

To explore the mechanisms that underlie these effects, we focused on GnRH neurons, a small subset of neurons that serve as master regulators of reproduction in mammals (Gore, 2002). GnRH neurons secrete gonadotro-

pin releasing hormone (GnRH), a peptide that stimulates the pituitary to release gonadotropins. The gonadotropins, in turn, act on the gonads to regulate the onset of puberty, estrus cycling, and gametogenesis. Brain injections of GnRH are reported to stimulate sexual behaviors, suggesting that GnRH neurons also participate in the neural circuits that control these behaviors.

To investigate the neural circuits that underlie pheromone effects on reproduction, we made transgenic mice in which the GnRH promoter drives the expression of two proteins, the transneuronal tracer, BL, and green fluorescent protein (GFP) (Boehm et al., 2005). Our idea was that GFP would be confined to GnRH neurons, but BL would travel to neurons both upstream (presynaptic) and downstream (postsynaptic) of GnRH neurons in one or more neural circuits. Downstream neurons would have GnRH containing axons in their vicinity whereas upstream neurons would not, allowing us to distinguish upstream and downstream neurons by immunostaining with antibodies against BL and GnRH.

In the transgenic mice, most or all GnRH neurons contained both GFP and BL. Only GnRH neurons expressed GFP, but BL was seen in many other neurons. GnRH neurons are confined to the anterior hypothalamus and adjacent areas. However, we detected BL+ neurons in many brain areas, some of which also had GnRH fibers and others that did not. Our studies indicate that the BL+ neurons in these animals are only one synapse away from GnRH neurons.

GnRH neurons have traditionally been thought of primarily as neuroendocrine cells that control reproductive hormones by their effects on the pituitary. However, our results indicate that GnRH neurons, which number about 800 in mice, communicate with about 50,000 other neurons located in 53 brain areas with diverse functions. About 10,000 neurons in 19 brain areas transmit signals to GnRH neurons, as many as 30,000 neurons in 28 brain areas receive signals from GnRH neurons, and at least 6 areas have bidirectional communication with GnRH neurons.

These findings indicate that GnRH neurons are exquisitely poised to integrate information from multiple different brain areas regarding the animal's internal state and its external environment. They further indicate that GnRH neurons are likely to influence a variety of brain functions, presumably coordinating those functions to optimize reproductive success. These studies lay a groundwork for future molecular studies to unravel the complex mechanisms that underlie reproductive physiology and behavior in mammals.

These studies revealed that GnRH neurons communicate with several brain areas that have been implicated in sexual behavior. These include seve-

ral areas of the hypothalamus as well as the medial amygdala, which receives pheromonal input derived from the VNO (Simerly, 2002). The identification of genes expressed in BL+ neurons in these brain areas could serve as a first step in identifying genes involved in sexual behaviors in mammals.

Surprisingly, we also found BL+ neurons in several areas of the olfactory cortex. Some of the labeled neurons were near GnRH fibers whereas others were not, suggesting that some of these neurons are upstream of GnRH neurons while others are downstream. We further found that pheromones that affect male or female reproductive function induced c-Fos in BL+ neurons in VNO-recipient brain areas as well as in certain parts of the olfactory cortex. Interestingly, we also saw double-labeled cells in one olfactory cortical area in response to a source of common odorants.

Together, these findings indicate that GnRH neurons receive pheromone signals from both the olfactory epithelium and VNO. Moreover, it is likely that GnRH neurons receive information regarding some common odorants. Finally, it appears that GnRH neurons can transmit signals to both VNO-recipient brain areas and the olfactory cortex and may therefore be able to modulate the processing of certain pheromone and/or odor signals in the brain. Since presenting this work as a lecture, we have published the discovery of a second class of chemosensory receptors in the olfactory epithelium that may be involved in the detection of social cues, such as pheromones, in the nose.

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ABSTRACT

The olfactory system of mammals can detect thousands of odorant molecules and often discriminate between nearly identical structures. In addition, a second chemoreception system is dedicated to the perception of pheromones, chemicals that mediate communication between individuals of the same species. Olfactory information, encrypted in

the structure of odorant molecules, is first decoded by receptors spanning the membrane of olfactory neurons. The electrical signals thus generated are conveyed to the olfactory bulbs and from there to the olfactory cortex to build an odor map, that generates a perception. At the level of the olfactory cortex a degree of signal processing takes place, modifying the original chemical information. We are thus beginning to understand how mixtures of odorants can produce novel sensations in humans.

RIASSUNTO

Il sistema olfattivo dei mammiferi è in grado di percepire migliaia di molecole odorose e spesso può distinguere strutture chimiche quasi identiche. Un secondo sistema di chemiorecezione è dedicato alla percezione dei feromoni, sostanze chimiche che mediano la comunicazione fra individui della stessa specie. L'informazione olfattiva, nascosta nella struttura delle molecole odorose, e' dapprima decodificata da parte di recettori che attraversano la membrana dei neuroni olfattivi. I segnali elettrici, così generati, vengono inviati ai bulbi olfattivi e da questi alla corteccia olfattiva, che genera una mappa dell'odore, riconosciuta dal cervello. A livello della corteccia olfattiva avviene una notevole elaborazione dei segnali, modificando sostanzialmente l'informazione chimica originaria. Cominciamo ora a comprendere perché miscele di odoranti possono generare nuove sensazioni nel cervello umano.

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Paolo Pelosi*

Odorant-binding proteins

introduction

The perception of odours involves the stimulation of specific olfactory receptors expressed on the dendritic membrane of sensory neurons. Olfactory receptors have been identified both in vertebrates (Buck and Axel, 1991) and in insects (Clyne et al., 1999; Vosshall et al., 1999) and in several cases their interactions with odorant molecules have been observed in heterologous systems (Krautwurst et al., 1998; Zhao et al., 1998; Wetzel et al., 2001; Hallem et al., 2004).

However, in order to reach the dendritic membrane, odorant molecules have to cross an aqueous barrier, the nasal mucus in vertebrates, the sensillar lymph in insects. In both cases these secretions contain small acidic proteins in extremely high concentrations. Such proteins have been named odorant-binding proteins (OBPs) based on their property of spocifically interacting with odour molecules. OBPs were discovered in the early eighties at about the same time in vertebrates (Pelosi et al., 1981, 1982) and in insects (Vogt and Riddiford, 1981). In both cases a ligand-binding approach was adopted, using the strong bell pepper odorant 2-isobutyl-3-methoxypyrazine to fish out the bovine OBP from an extract of nasal tissue, and the specific pheromone, hexadecadienyl acetate, to identify the pheromone-binding protein from the antennae of the moth *Antheraea polyphemus*.

Despite their common name, OBPs of vertebrates and those of insects belong to separate families, different in their amino acid sequences and in their three-dimensional folding.

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In insects a second family of binding proteins, named chemosensory proteins (CSPs) fills the lymph of chemosesilla. Like OBPs, also CSPs reversibly bind small molecules, such as pheromones, odorants and tastants and are believed to perform roles similar to OBPs.

Several hundreds OBPs and CSPs have been identified and their number is rapidly increasing, thanks to the information available for sequenced genomes. For several members structural information is available, as well as binding specificity to chemostimulats and patterns of expression in different organs, sexes, castes and during the life cycle.

Recent reviews have summarised the most relevant information on odorant-binding proteins in vertebrates and in insects (Pelosi, 1994, 1996, 1998, 2001; Steinbrecht, 1998; Tegoni et al., 2000; Picimbon, 2003; Vogt, 2003, 2005; Leal, 2005; Pelosi et al., 2006).

Although a great amount of structural and functional information is available for OBPs, their specific role in odour and pheromone perception is still unclear. However, their extremely high concentration around olfactory dendrites (in the sensillar lymph of insects their concentration has been estimated to be close to 10 mM) and the relatively high amount of energy spent for their synthesis strongly indicates that these proteins are important for the survival of the individual or for the propagation of the species.

ODORANT-BINDING PROTEINS OF VERTEBRATES

In vertebrates OBPs have been identified and purified from several mammalian species (cow, pig, rabbit, mouse, rat, porcupine, etc.) and two amphibians (frog and *Xenopus*). Attempts to identify odorant-binding proteins both from fishes and birds failed. Moreover, a search in the genomes of the zebrafish and the pufferfish could not yield any sequence similar enough to the known OBPs.

In humans a couple of closely related genes encoding OBPs have been described (Lacazette et al., 2000) and the protein corresponding to one such isoform has been detected in the nasal mucus (Briand et al., 2002). However, their concentration is much lower than in other vertebrates.

OBPs of vertebrates belong to a very large superfamily of binding proteins, called lipocalins (Flower et al., 2000). They include several carrier proteins, such as retinol-binding protein, dedicated to transport hydrophobic

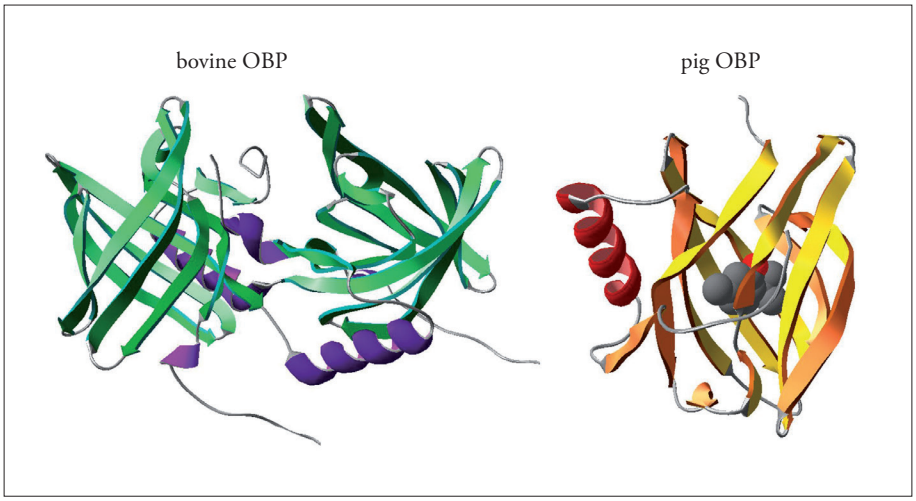

Fig. 1 *Three-dimensional structures of bovine (Bianchet et al., 1996 Tegoni et al., 1996) and pig (Vincent et al., 2000) odorant-binding proteins. Both proteins are mainly present as β-sheets domains folded into the typical β-barrel motif of lipocalins. The bovine protein present the unusual phenomenon of "domain swapping", that likely stabilises the structure of this proteins that lacks cysteines and therefore cannot establish disulphide bridges. The pig OBP is shown complexed with a molecule of thymol*

compounds across aqueous media in the body and β-lactoglobulin, one of the major proteins of milk.

The three-dimensional structure of vertebrates' OBPs reproduces the "β-barrel" motif, common to all lipocalins and constituted by eight antiparallel β-sheets assembled in a sort of compact basket, and a short segmant of α-helix. A hydrophobic cavity, defined by the eight β-sheets can accept organic molecules of medium size, such as odorants and pheromones. The bovine OBP was the first protein of this family to be crystallised (Bianchet et al., 1996; Tegoni et al., 1996). It presents the unusual phenomenon of domain-swapping, where in the homodimeric structure the C-terminus of one monomer overlaps with the main domain of the other monomer. This greatly contributes to stabilising the structure of this protein, that, unlike other OBPs, does not contain any cysteine and therefore cannot form disulphide bridges. The pig OBP, on the other hand, that has also been extensively studied at the structural level, is a monomer and its folding is stabilised by an internal disulphide bridge. Both the bovine and the porcine OBPs have been also used to obtain cristals of the protein complexed with various ligands in order to visualise the position of the ligands in the binding pocket (Vincent et al., 2000, 2004). Interestingly, these proteins

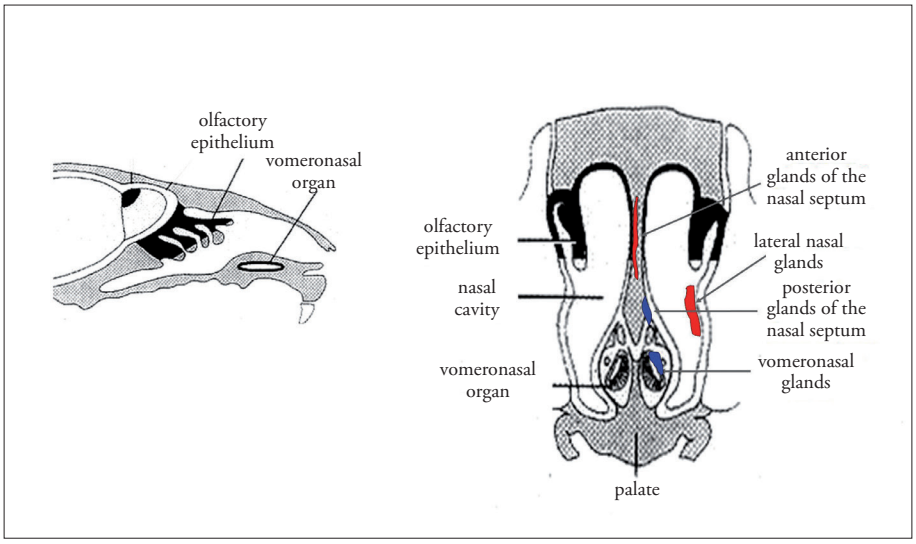

Fig. 2 *Sections of the nasal cavity in the rat, showing the olfactory and vomeronasal areas and the location of glands producing OBPs. OBP1 (red) and OBP2 (blue) are expressed in different glands and in different periods of life. (Ohno et al., 1996). In any case, synthesis of OBPs occurs in the vomeronasal organ or in the respiratory epithelium, but not in the olfactory region*

can accept several molecules of different structure each of them sitting in the binding cavity with a different orientation. As an example, figure 1 reports the three-dimensional structure of bovine OBP complexed with a molecule of a selenazole derivative and the pig OBP complexed with a molecule of thymol.

The main unaswered question about OBPs concerns their physiological function. Twenty five years after their discovery, this is still a matter of debate. To address this problem, several binding studies have been performed, using different classes of odorant molecules (Dal Monte et al., 1991, 1993; Herent et al., 1995; Loebel et al., 1998; Paolini et al., 1998, 1999). The conclusion of such experiments is that OBPs present a broad specificity of binding, accepting molecules of medium size, but different in molecular shape and in odour quality. In some cases, it has been reported that different OBPs from the same animal species present different spectra of binding, although with poor specificity (Loebel et al., 2002).

Another element indicating that OBPs might not be involved in odour perception is the observation that these proteins are not synthesised in the olfactory epithelium, but in glands of the respiratory and vomeronasal areas. Moreover, given the anatomy of the olfactory system, OBPs cannot travel from their sites of production to the olfactory area, but can

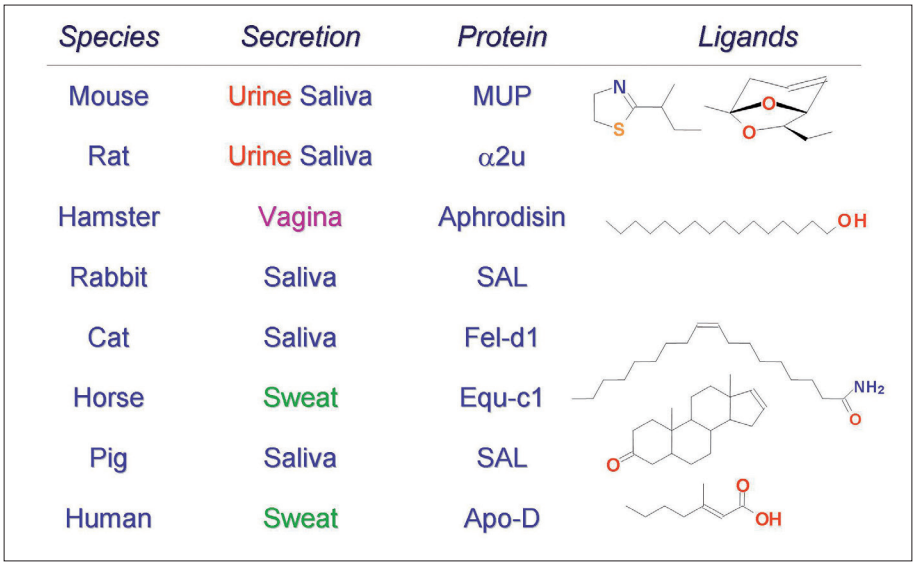

Fig. 3 *Lipocalins involved in chemical communication. Proteins very similar or even identical to OBPs are secreted in biological fluids, such as urine, saliva, vaginal discharge and sweat, utilised by some species to deliver their pheromones in the environment. Such lipocalins have been shown in some cases to carry as endogenous ligands molecules recognised or hypothesised to be specific pheromones*

be rather pumped into the vomeronasal organ (Ohno et al., 1996, Pes et al., 1998). Figure 2 shows a section of the nose cavity in the rat with the positions of the different sensory epithelia and the glands where OBPs are synthesised.

Such findings suggested the hypothesis that OBPs could play a role in pheromone rather than in odour perception. This idea is further supported by the close structural similarity of OBPs with proteins secreted in different parts of the body and involved in the delivery of pheromones.

Such proteins are themselves lipocalins, very similar and in some cases identical with OBPs in their amino acid sequence and three-dimensional folding (Cavaggioni et al., 2000). They are abundantly present in body fluids, such as urine, saliva, sweat and vaginal discharge (Singer et al., 1986; Cavaggioni et al, 1990; Zeng et al., 1996; Marchese et al., 1998; Briand et al., 2000; D'Innocenzo et al., 2006). These secretions have been reported in some mammals to contain specific pheromones and are indeed used in some species to send signals to individuals of the other sex, to mark territory or other sorts of chemical communication (Hurst et al., 2001). Figure 3 lists

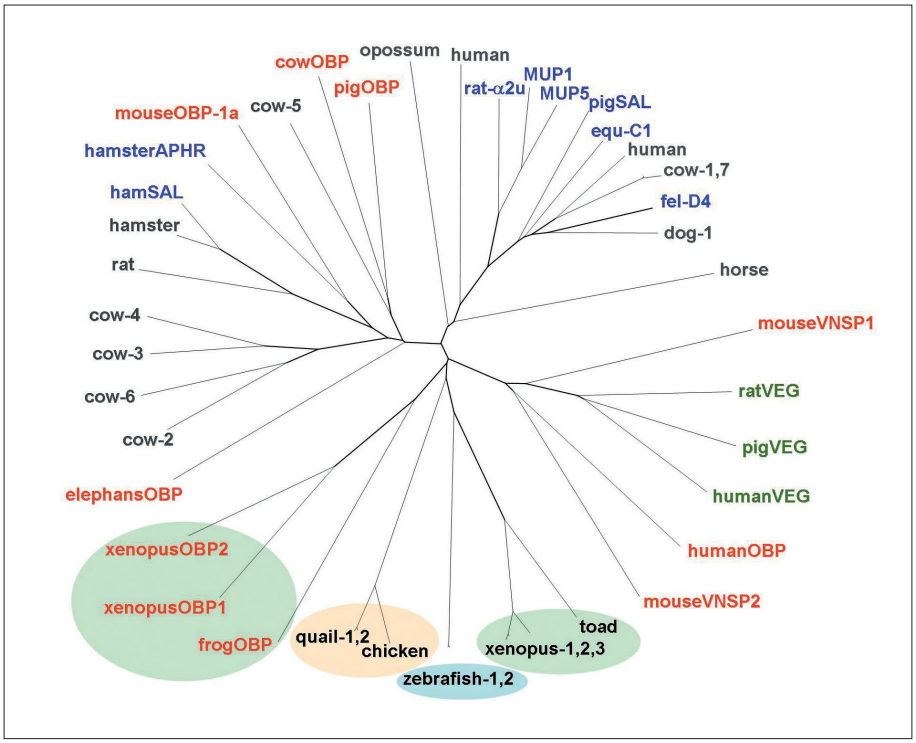

Fig. 4 *Graphical representation of sequence similarities between vertebrates OBPs and other lipocalins involved in chemical communication. OBPs are indicated in red, pheromone carriers in blue, VEG proteins in green and others in grey*

some of these proteins and the fluids that contain them. It is remarkable and interesting that in some cases, such as in the mouse urinary proteins (MUPs) or in the pig salivary proteins (SALs), endogenous ligands have been identified inside the binding cavity of the protein, when purified from urine or saliva (Bacchini et al., 1992; Robertson et al., 1993; Marchese et al., 1998). These ligands had previously been recognised as specific pheromones for the species. The boar sex pheromones, for instance, androstenone and androstenol, are complexed with the two isoforms of SAL expressed in the saliva of male pig. Moreover, the expression of these proteins is sex-specific, being present only in the urine of mature male mice and in the saliva of the boar, but not in the sow. Quite interestingly, the same proteins are also found in the nasal mucosa: in this case they are present in both sexes and are devoid of ligands (Scaloni et al., 2001).

It is not surprising that chemical communication in mammals utilises the same proteins for releasing pheromonal messages in the environment and for

receiving them, just like a radio transmitter and receiver tuned on the same wavelength.

The close relationship between OBPs and other lipocalins involved in chemical communication is clear from figure 4, a graphical representation of sequence similarities, where there is no clustering of such subgroups of lipocalins. In fact the distinction is based on the site of production of such proteins, rather than on their structural elements. A third class of lipocalins is also present among the sequences of figure 4. They are called VEG from the von Ebner glands of tongue's papillae, where they were first identified (Schmale et al., 1990). These proteins, proposed at the beginning to be involved in taste, have been later found in lachrymal glands and in other secretion and are now believed to perform an antibacterial role (Scalfari et al., 1997; Redl, 2000).

ODORANT-BINDING PROTEINS AND CHEMOSENSORY PROTEINS OF INSECTS

In insects two families of small soluble proteins surround the dendrites of chemosensory neurons (Pelosi, 1998; Steinbrecht, 1998; Picimbon, 2003; Vogt, 2003, 2005; Leal, 2005 Pelosi et al., 2006). Proteins of the first family are about 140-150 amino acid long and are called OBPs by analogy with vertebrates' OBPs, although there is no similarity between the two classes of proteins. The second group (CSPs) comprises polypeptides of 100-120 amino acids, again completely different in sequence and structure from both OBPs of insects and vertebrates.

A typical signature of insects' OBPs is the presence of six cysteine residues in conserved positions and connected by three interlocked disulphide bridges. CSPs, on the contrary, present only four cysteines with two disulphide bonds between neighbouring residues. Both types of proteins are mainly folded in α-helical domains, although with different overall structures. Both OBPs and CSPs present hydrophobic binding cavities and have been shown to reversibly bind several organic molecules of medium size.

A great number of sequences for both OBPs and CSPs are available in the database, mainly thanks to recent genome information. At the beginning, most of the experimental work concentrated on moths, because of the wide information already available on their pheromones and of the large size of some species, that made biochemical work more feasible. In Lepidoptera four types of OBPs have been distinguished, pheromone-binding proteins (PBPs), so called because they specifically bind pheromones (Vogt and Riddiford,

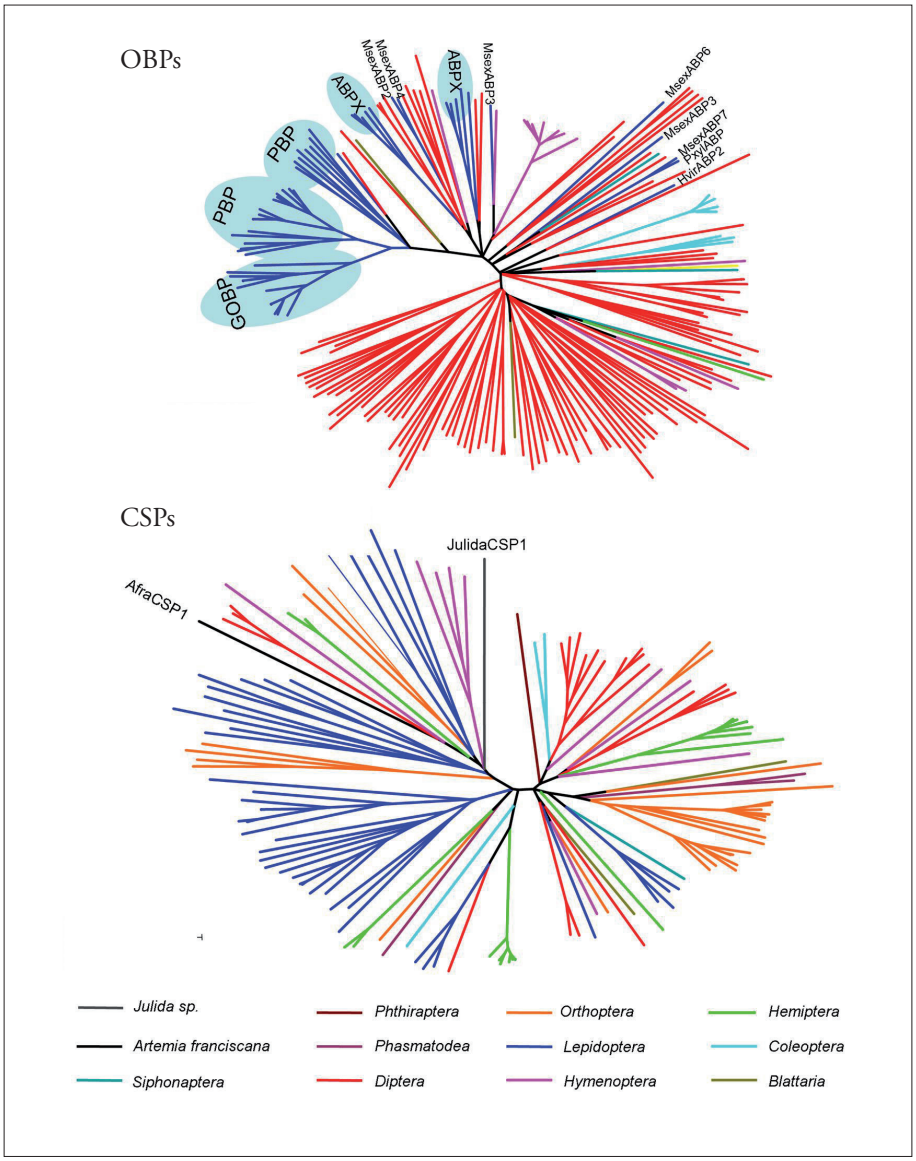

Fig. 5 *Graphical representation of sequence similarities between insects OBPs (upper graph) and insects CSPs (lower graph). Colours indicate different Orders*

1981), two "general odorant-binding proteins" (GOBP1 and GOBP2) (Vogt et al., 1991a, 1991b) and "antennal binding protein X" (ABPX) (Krieger et al., 1996). All of them are referred to as OBPs.

Later, first with the applications of molecular biology techniques and then with the accessibility of genome sequences, other orders of insects provided the species of choice, such as *Drosophila*, *Anopheles* and more recently the honeybee *Apis mellifera*. So far OBPs and CSPs have been reported in more than 40 species of insects, belonging to 10 different Orders. In species other than Lepidoptera the distinction of OBPs into the above mentioned four classes is not feasible. In fact, OBPs are very divergent across evolution and often share not more than 10-15% of their residues between different species (McKenna et al., 1994; Pikielny et al., 1994). This low degree of similarity is also observed between sequences of the same species, such as in *Drosophila* (Galindo and Smith, 2001; Graham and Davies, 2002; Hekmat-Scafe et al., 2002; Zhou et al., 2004a). In fact, in some cases the only conserved motif is a pattern of six cysteines, that in the three-dimentional structure are connected by three interlocked disulphide bridges (Leal et al., 1999; Scaloni et al., 1999;). The availability of genomes has allowed to determine how many OBPs are expressed by an insect species. In *D. melanogaster* there are 39 genes encoding "classical OBPs" with the typical six-cysteine motif, and 12 that contain, in addition to the six-cysteine signature, more cysteine residues (Hekmat-Scafe et al., 2002; Zhou et al., 2004a). The genome of *Anopheles gambiae* also contains a great number of genes encoding putative OBPs: 37 "classical" sequences and 35 with additional motives (Biessmann et al., 2002; Vogt, 2002; Xu et al., 2003; Zhou et al., 2004a). On the other hand, only half a dozen OBPs have been reported in the honeybee *Apis mellifera* and in the silkmoth *Bombyx mori*. For CSPs also, the scattered information available suggests that different species might be endowed with different numbers of genes encoding such proteins. It is important to remember that these data are mainly the result of bioinformatic investigation, while we still know very little whether all these proteins are expressed, or if they are really involved in chemoreception. As an example, the protein p10 of the cockroach, that shares all the structural characteristics of CSPs, has been shown to be produced during limb regeneration and is likely involved in such process (Kitabayashi et al., 1998).

Figure 5 reports in a graphical representation the structural relationships between most of the OBPs and CSPs described. It is interesting to observe that, while OBPs have likely appeared with insects, as they have not been found so far in other arthropods, CSPs seem to have an earlier origin (Pelosi et al., 2006). In fact, at least two sequences, significantly similar to CSPs of insects have been reported in a crustacean (*Artemia franciscana*) and a millipe-

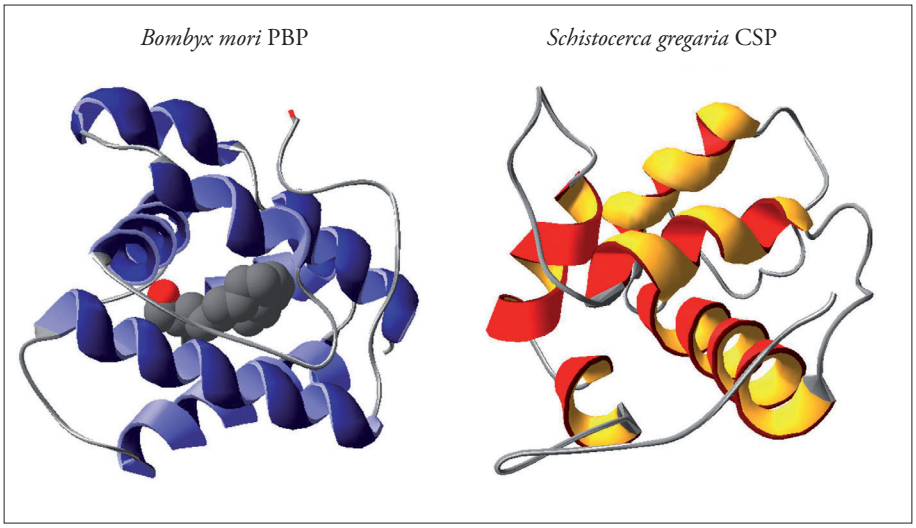

Fig. 6 *Three-dimensional structures of representative OBP and CSP. On the left the PBP of Bombyx mori complexed with a molecule of the specific sex pheromone, bombykol (Sandler et al., 2000). On the right the structure of one of the CSPs of the desert locust Schistocerca gregaria (Tomaselli et al., 2006). In both cases the proteins are mainly present in α-helical domains, but folded in different fashions.*

de (*Julida sp.*). However, a role in chemoreception for these proteins has not been demonstrated and they might well perform different functions.

The three-dimensional folding of insects OBPs and CSPs has been studied by X-ray diffraction and NMR. So far the structures of six OBPs and two CSPs are available (Sandler et al., 2000; Horst et al., 2001; Lartigue et al., 2002; Lee et al., 2002; Campanacci et al., 2003; Kruse et al., 2003; Lartigue et al., 2003; Lartigue et al., 2004; Mohanty et al., 2004; Mosbah et al., 2004; Tegoni et al., 2004; Wogulis et al., 2005; Tomaselli et al., 2006). Both classes of proteins mainly present α-helical domains, but with different and unique types of folding. The structure of OBPs is stabilised by three conserved interlocked disulfide bridges, while in CSPs two bonds between adjacent cysteines do not apparently contibute to the stability of the protein. However, both OBPs and CSPs are very stable proteins and can recover their complete activity after being heated to 100°C for several minutes.

As representative examples of proteins of the two classes, figure 6 reports the three-dimensional structures of the *Bombyx mori* PBP complexed with a molecule of bombykol, the specific sex pheromone (Sandler et al., 2000) and that of one of the CSPs of the desert locust, *Schistocerca gregaria* (Tomaselli et

al., 2006). The PBP of *B. mori* has been shown to undergo a conformational change when the pH is lowered from 6.5 to 4.5: the C terminus, unstructured at physiological pH, folds into an α-helical segment in acidic conditions and fills the binding cavity, thus chasing out the pheromone molecule (Horst et al., 2001). Such mechanism might be general for insects' OBPs and probably triggered in some proteins by the ligand. This could be the case for LUSH, one of the OBPs of *Drosophila*. Its structure shows the C-terminus folded back into the core of the protein (Kruse et al., 2003). Such conformation is similar to that assumed by the moths' PBPs in acidic conditions, but occurs in this protein at neutral pH. Based on the good affinity of this protein to large aromatic molecules, it has been proposed that in order to let such ligands enter the binding cavity, the C-terminus of the protein should move out, with a mechanism similar to that described for the *B. mori* PBP (Zhou et al., 2004b).

Also CSPs apparently undergo conformational changes, as in the case of the *Mamestra brassicae* protein, that can bind three molecules of 1-bromododecanol, sensibly swelling in this process (Campanacci et al., 2003). Conformational changes occurring in OBPs as a consequence of ligand binding could be important for understanding the function and the mode of action of these proteins in the process of odour perception.

In insects the anatomy of the chemoreception systems has greatly helped understanding the function of OBPs and CSPs. In fact, while in vertebrates the nose performs different functions (olfaction, pheromone perception, other sensing modalities and respiration) and houses different types of glands, in insects morphologically different sensilla are dedicated to detecting specific semiochemicals in the environment. Electrophysiological recordings can identify the chemical stimuli for each type of sensilla (Kaissling et al., 1985), while electron microscopy has shown that different classes of sensilla often contain different OBPs or CSPs. Thus, for instance, The PBP of *B. mori* is specifically expressed in sensilla trichodea, that selectively respond to the sex pheromone (Steinbrecht et al., 1992, 1995; Maida et al., 1993), thus giving physiological significance to the observation that this protein binds bombykol. On the other hand, sensilla basiconica, that respond to general odours have been shown to express GOBPs (Laue et al., 1994).

Moreover, the morphology of chemosensilla is rather simple, generally a cone-shaped protuberance of the cuticle, that hosts the dendrites of one or more olfactory or taste neurons. The space between the external cuticular wall and the dendrites is filled with a lymph, that contains as major components

OBPs or CSPs. These proteins are present at extremely high concentrations, estimated around 10 mM (Klein, 1987).

Based on these observations, the involvement of OBPs and CSPs of insects in chemosensing processes appears more clear and convincing than in the case of vertebrates, where the presence of several chemosensing modality, together with other physiological functions in the same anatomical cavity makes the system more difficult to study. Consequently, most of the recent work on OBPs has taken insects as models to investigate their physiological function.

Very recently, evidence that these proteins are required for olfaction has been provided, with regard to the already mentioned *Drosophila*'s protein LUSH. It has been demonstrated, in fact, that silencing the gene for this protein has the effect of suppressing both the electrophysiological and the behavioural response to the male pheromone vaccenyl acetate. Reactivation of the gene or even reinsertion of the protein in the sensillum restores normal responses (Xu et al., 2005). More recently, the same Authors have demonstrated that inactivating the synthesis of the receptor in the same cells also suppresses response to the pheromone (Ha and Smith, 2006).

It seems therefore that both receptor and binding protein are required for a correct functioning of the olfatory system, but the specific role of OBPs in the all process has still to be clarified.

RIASSUNTO

Le proteine leganti gli odori (OBPs: Odorant-binding proteins) sono piccoli polipeptidi di natura solubile che si pensa siano coinvolte nella percezione olfattiva. Tuttavia, la loro funzione specifica e' ancora poco chiara, venticinque anni dopo la loro scoperta. La identificazione dei recettori olfattivi e delle vie nervose che collegano i neuroni periferici alle aree del cervello ha permesso di comprendere la maggior parte degli elementi biochimici che permettono di convertire i messaggi chimici delle molecole odorose in sensazioni, emozioni, reazioni comportamentali. Le OBP restano al di fuori di questo quadro, quasi come elementi non necessari. Tuttavia, le eccezionali concentrazioni nelle quali esse si trovano presenti e la loro grande varieta' indicano un ruolo importante nel processo della comunicazione chimica. In questo lavoro vengono presentate le caratteristiche strutturali delle OBP e le loro affinita' verso molecole odorose e feromoni. Lo stesso nome di OBP indica due classi di proteine di struttura diversa. Le OBP dei vertebrati appartengono alla superfamiglia delle lipocaline, proteine di trasporto di composti idrofobici in fluidi biologici acquosi, che condividono una compatta struttura costituita da foglietti beta. In quelle degli insetti, invece, sono presenti per la maggior parte segmenti di α-elica. Una terza classe di piccole proteine, denominate CSP (chemosensory proteins), presenti solo negli insetti, potrebbe essere coinvolta nella comunicazione chimica. Tutti questi tre tipi

di polipeptidi sono estremamente stabili, come si richiede a proteine che esplicano la loro funzione all'interfaccia fra il sistema olfattivo e l'ambiente esterno.

ABSTRACT

Odorant-binding proteins (OBPs) are small soluble polypeptides that are believed to be involved at some stage in olfactory perception. Their specific role, however, is still unclear twenty five years after their discovery. The identification of olfactory receptors and the mapping of the neural network connecting the periphery of the olfactory system to different areas of the brain has clarified most of the biochemical steps converting the messages encoded in the structure of odorant molecules into sensations, emotions, behavioural responses. OBPs still remain out of this picture, as if they were unnecessary elements. However, their exceptionally high concentration and their wide variety points to an important function in chemical communication. Here the structural elements of OBPs are reviewed, together with their binding properties towards odorants and pheromones. The same name (OBPs) indicates two classes of structurally different proteins. Those of vertebrates belong to the superfamily of lipocalins, carrier proteins for hydrophobic compounds in aqueous biological fluids sharing the typical β-barrel structure. Those of insects, instead, are mainly folded in α-helical domains. A third group of small proteins, only expressed in insects and named CSPs (chemosensory proteins) are also believed to be involved in chemical communication. All these three types of polypeptides are extremely stable, as it seems appropriate for proteins located at the inteface between the olfactory system and the external environment.

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Plasticity of the olfactory system

INTRODUCTION

Up to the last two decades, the term 'neurogenesis' was used under its original, embryological meaning, that is 'genesis of the nervous system', and not under the meaning of 'genesis of neurons', since it was thought that no new neurons could be generated after the accomplishment of development. Although some proliferative activity was firstly detected in the brain by using tritiated thymidine DNA incorporation as a marker of cell division (Altman & Das, 1965; Altman, 1969), the issue of adult neurogenesis was considered with scepticism (Rakic, 1985). In the following years, comparative studies demonstrated interesting phenomena of adult neurogenesis in different non-mammalian species, such as songbirds (Alvarez-Buylla and Nottebohm 1988; Alvarez-Buylla and Kirn, 1997), thus leading to the assumption that striking structural plasticity might be restricted to certain vertebrate classes.

A profound change in this vision occurred in the first '90s, starting from two simultaneous findings: the first isolation of adult neural stem cells (Reynolds & Weiss, 1992; Morshead et al. 1994) and the occurrence of a massive cell migration toward the postnatal and adult mammalian olfactory bulb, involving neuroblasts continuously-generated into the subventricular zone (SVZ; Lois & Alvarez-Buylla, 1994; Luskin, 1993). Both these studies strengthened the idea that striking structural plasticity, including the genesis of new neurons, do actually exist in the adult mammalian brain, thus opening a new series of researches focussed to a deeper understanding of these processes.

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Here we summarize the current knowledge on adult neurogenesis, emphasizing on the anatomical substrate and molecular factors regulating neurogenesis in the SVZ-OB system.

SITES OF ADULT NEUROGENESIS

In vivo under physiological condition, constitutive neurogenesis is thought to be restricted to two main regions: the SVZ-OB system (Alvarez-Buylla and Garcia-Verdugo, 2002) and the hippocampal subgranular zone (Kempermann and Gage, 2000) (fig. 1A). Notably, comparative studies demonstrated that adult neurogenesis and the existence of progenitor stem-like cells occur in several mammalian species human included (Eriksson et al., 1998; Sanai et al., 2004). Whereas in both systems a strong selection of the newly generated neurons does occur (Petreanu and Alvarez-Buylla 2002; Gould et al., 1999b), a significant percentage of the newly generated cells is still present after several months from their genesis and show mature phenotypes and electrophysiological activity (Petreanu and Alvarez-Buylla 2002; Cameron and Mckay 2001; Belluzzi et al., 2003) indicating functional integration in the mature pre-existing circuits.

Interestingly, studies performed in rodents, primates, and lagomorphs indicate that genesis of new neurons can also be detected under physiological conditions in other brain regions such as the visual cortex (Kaplan 1981), neocortex (Gould et al., 1999, Dayer et al. 2005), amigdala (Bernier et al., 2002) and striatum (Dayer et al. 2005; Luzzati et al., 2006) (fig. 1A). New evidences also indicate that following certain forms of brain injury, neural progenitor cells can give rise to new neurons in regions of the adult CNS classically considered as "non neurogenic" (see Emsley et al., 2005, for review). Nevertheless, in these regions, both physiological and induced neurogenesis ends up with the integration of very few mature neurons compared to SVZ and hyppocampal neurogenesis, and their functional role, if exists, has not been so far identified.

This increasing body of work, besides investigating the sites and the functional implications of neurogenesis in the adult brain, also investigates the potentiality of the putative stem cell compartments to be activated and directed towards production of specific neuronal cells. Thus, adult neurogenesis provides a model to understand physiological mechanisms of neural development in the mature brain, but also opens the possibility that stimulation of this process can be used to promote therapeutic strategies for CNS diseases.

THE SVZ-OB SYSTEM AS A MODEL TO STUDY ADULT NEUROGENESIS

Integration of newly generated neurons in adult pre-existing circuits require a precise sequence of steps including: proliferation of progenitor stem-like cells, production of transit amplifying cells, genesis of neuroblasts, migration from the origin site to the final target, morphological and functional maturation. All these stages are regulated by a complex interplay between genetic and epigenetic influences, which lead the progression of neuronal progenitors toward functional mature neurons. Only a deep knowledge of these mechanisms will improve our capacity to modulate adult neurogenesis. In this context, the SVZ-OB system offers unique opportunities to precisely dissect molecular mechanisms regulating neuronal birth, migration and differentiation. Indeed, newly formed cells of the olfactory bulb are generated from the proliferative activity of multipotent neuronal progenitor cells residing in the SVZ of the lateral ventricle and its rostral extension, then migrate along the rostral migratory stream to the OB where they finally differentiate into interneurons (Peretto et al., 1999). Studies performed during the last ten years besides clearly define the anatomical organization of the adult (Peretto et al., 1997; Doetsch et al., 1997) and postnatal SVZ (Peretto et al., 2005), have demonstrated that neurogenic activity in the SVZ-OB system is dynamically regulated by diverse molecular and environmental factors (Alvarez-Buylla and Garcia-verdugo 2002 for review). In particular, it has been suggested an important role of the SVZ glial cells, which are involved in providing a stem cell niche (Lim et al., 2000; Peretto et al., 2002, 2004) and the stem cell reservoir in this brain region (Doetsch et al., 1999). Moreover, the process of selection/recruitment of new neurons in the OB is highly regulated in an activity dependent manner, supporting the idea of a functional role of newborn neurons. Thus, the SVZ-OB system represents a unique model to investigate phenomena of neuronal plasticity and in particular neurogenesis in the adult mammalian brain.

STRUCTURAL ORGANIZATION OF THE ADULT SVZ

The adult SVZ or subependymal layer, represents a remnant of the embryonic germinative matrices. It persists during adulthood, as a layer of dividing cells, in the forebrain periventricular region and along its rostral extension towards the olfactory bulb (fig. 1A). The anatomy and cell biology of the SVZ have been clarified only recently. In light microscopy, the SVZ appears as a mass of

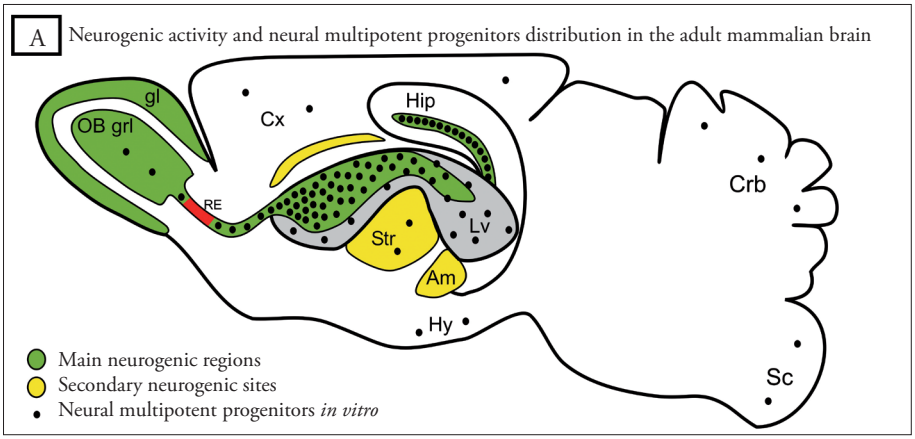

Fig. 1A *Neurogenic activity and neural multipotent progenitors distribution in the adult CNS of mammals. Schematic representation of a medial parasagittal section of the adult rodent brain. The SVZ-OB system and the hippocampus (in green) represent the two main areas showing protracted neurogenesis. Neurogenic activity has been also described in both physiological and pathological conditions, in different mammalian species, in regions such as the amigdala, corpus striatum and deepest layers of cerebral cortex (in yellow). Black dots indicate the distribution of adult neuronal progenitors obtained by in vitro studies.*
Am, amygdale; *Crb, cerebellum; Cx, cerebral cortex; Hip, hippocampus; Hy, hypothalamus; Lv, lateral ventricle; OB, olfactory bulb; RE, rostral extension; Sc, spinal cord; Str, striatum; gl, glomerular layer; grl, granule layer.*

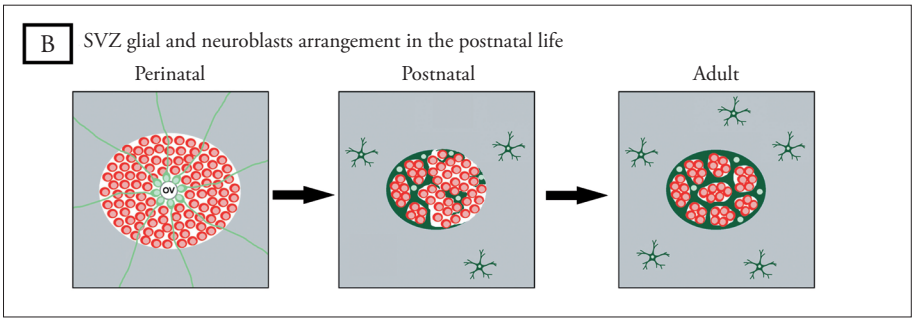

Fig. 1B *Morfo-anatomical modifications occurring during the first three postnatal weeks of life in the SVZ-OB system. Pictures represent three coronal sections cut at the level of the SVZ-RE (circle red in A) at different postnatal stages. Radial glial cells with cell bodies in contact with the olfactory ventricle (OV) shift to atrocyties which, in the SVZ, progressively originate the glial tubes. In parallel the homogeneous mass of migrating neuroblasts clusterizes in chains.*

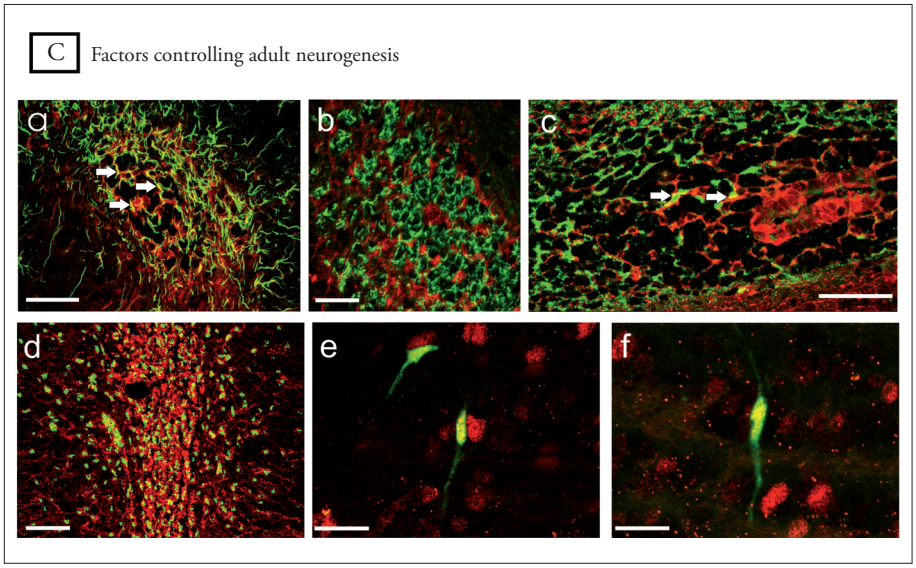

Fig. 1C *Factors controlling adult neurogenesis. a-c) Noggin is expressed in the SVZ-glial compartment of the adult SVZ-RE of mice (coronal sections). Noggin protein (red) is mostly localized in the GFAP-positive (green) SVZ glial cells (yellow staining and arrows in a). b) Noggin is not expressed by the PSA-NCAM positive migrating neuroblasts (green). c) A partial degree of colocalization (arrows) in the SVZ glial compartment occurs between Noggin (red) and BMP4 (green). d-f) p-CREB expression in the SVZ migrating neuroblasts. d) coronal section of the SVZ-RE cut at the level of the OB. p-CREB expression (green) is abundant in the mass of DCX-positive (red) migrating neuroblasts reaching the OB. e-f) The green cells are migrating neuroblasts in the OB showing p-CREB expression in the nucleus (yellow) 15 days after their staining with the cell tracer CTG in the LV.*
Reference bars: *a, d, c, 50 µm; b, e, 20 µm; f, 10 µm.*

small, tightly-packed cells, easily recognizable from the surrounding mature brain parenchyma (Peretto et al., 1999). The exact nature and the mutual relationships of these cells were unraveled after detailed ultrastructural and immunocytochemical characterizations (Lois & Alvarez-Buylla, 1994; Lois et al., 1996; Jankovski & Sotelo, 1996; Peretto et al., 1997; Doetsch et al., 1997). The SVZ region of adult rodents contains at least four cell types: i) undifferentiated, bipolar-shaped cells showing the features of *migrating neuroblasts*, referred to as type A, ii) *astrocytes,* or type B cells, iii) *transit amplifying cells,* or type C, iv) *ependymal cells*, or type E. Type A cells are characterized by a bipolar-like aspect, with a leading and a trailing process, a large nucleus and a thin rim of electrondense cytoplasm, due to the abundance of many

free ribosomes. During migration toward the OB, neuroblasts are firstly organized as chains. Once they reach the core of the OB they migrate as single elements to the granule and glomarular layers (Bonfanti & Theodosis, 1994; Rousselot et al., 1995). Type B cells are considered as a particular type of protoplasmic astrocytes (Peretto et al., 1997). These cells are easily detectable by using immunocytochemical glial markers, such as the glial fibrillary acidic protein (GFAP), and for their watery cytoplasm at the ultrastructural level. They form a dense meshwork throughout the SVZ area, which is organized as longitudinally-oriented channels called *glial tubes* which enwrap the migrating neuroblasts (Lois et al., 1996; Jankovski & Sotelo, 1996; Peretto et al., 1997). This channel-like organization of the glia in the SVZ, along with the well known role that glial cells play in neuronal migration during CNS development (Rakic, 1988, 1990), has initially suggested the existence of a new type of gliophilic migration in the adult brain (Lois et al., 1996; Peretto et al., 1997). Nevertheless, since the tangential migration can also occur, *in vitro,* in absence of any glial substrate (Wichterle et al., 1997) and during the early postnatal life, when the glial tube organization is still absent (Peretto et al., 2005), the role of glial cells in SVZ migration so far is not completely clarified. Type C cells, have been described in adult mainly in the SVZ-LV and show ultrastructural features between type A and B cells. These cells represent the most actively proliferating cells in the SVZ and often are found organized as clusters juxtaposed to the type A cells (Doetsch et al. 1997). A monolayer of ependymal cells, with many long cilia, separates the SVZ from the ventricular cavity.

Thus, from the lateral ventricle to the center of the OB two main compartments can be sharply identified both morphologically and immunocytochemically: the glial tubes and the chains of migrating neuroblasts (for review, see Peretto et al., 1999). Apart from the markers GFAP and S-100, which identify virtually all astrocytes of the mature CNS (Peretto et al., 1997), the glial cells forming the glial tubes also express peculiar proteins (Peretto et al., 2005) such as, the cytoskeletal proteins vimentin, which is also abundant in immature glial and neuronal cell populations, ependymal cells, Bergmann and radial glia (Pixley & DeVellis, 1984; Peretto et al., 1997), and nestin, usually expressed in highly undifferentiated neuroepithelial cells but absent in most of the adult CNS (Peretto et al., 1999). The presence of the intermediate filaments vimentin and nestin in the glial tubes astrocytes, as well as the frequent detection of punctate glycogen granules, lead to consider them as a specialized subpopulation of glial cells sharing some features with embryonic radial glia (Jankovski &

Sotelo, 1996; Peretto et al., 1997, 1999). Indeed, SVZ glial cells also express the glial specific glutamate transporter GLAST (Bolteus and Bordey, 2004) and the brain-lipid-binding protein (BLBP) (Peretto et al., 2005). Both these antigens are present in subpopulations of mature glial cells and in the radial glia (Hartfuss et al., 2001). Thus, consistent with their complex role in regulating adult neurogenesis (see below) SVZ astrocytes show unique molecular characteristics. The chains of migrating neuroblasts can be specifically identified using antibodies raised against the polysialylated form of the neural cell adhesion molecule or *PSA-NCAM* (Bonfanti & Theodosis, 1994; Rousselot et al., 1995), which has been demonstrated to play a role in cell migration in this system (see Alvarez-Buylla and Garcia Verdugo 2002 for review), the neuron-specific class III beta-tubulin, highly expressed in young neuroblasts (Menezes & Luskin, 1994), the phosphoprotein stathmin (Camoletto et al., 1997), whose function remains unknown, and the microtubule binding protein doublecortin (Yang et al., 2004).

MORPHOLOGICAL AND MOLECULAR CHANGES OCCURRING IN THE SHIFT FROM POSTNATAL TO ADULT SVZ

Although the SVZ is an area of the adult brain retaining features characteristic of the embryonic brain, substantial differences in the morphological and molecular composition of its perinatal and adult counterpart have been recently described (Alves et al., 2002; Pencea and Luskin, 2003; Tramortin et al., 2003; Peretto et a., 2005). For instance, in the prenatal and early postnatal brain the LV continues anteriorly into an olfactory ventricle which closes after birth in rodents, giving rise to the SVZ rostral extension (-RE). The most striking differences involve the types and spatial distribution of glial cells and modes of SVZ neuroblasts migration (fig. 1B).

As above mentioned, SVZ astrocytes of the glial tubes originate form the embryonic radial glia (Gaiano et al., 2000; Alves et al., 2002; Tramortin et al., 2003), which represent the most abundant glial cell type of the embryonic SVZ (Misson et al., 1991; Rakic, 2003). According to the stem cell role recently proposed for SVZ astrocytes (Doetsch et al., 1997, see next paragraph), radial glia is able to give rise both astrocytic and neuronal precursors (Malatesta et al., 2000; Noctor et al., 2001) before undergo transformation into mature astrocytes (Misson et al., 1991). Detailed ultrastructural and immunocytoche-

mical analyses have clearly demonstrated that in mice and rats glial tube assembly and chain formation are the result of complex modifications occurring in the SVZ during the first three postnatal weeks of life (Peretto et al., 2005). In particular, during the first week after birth, although a great number of small glial processes are detectable, the migrating neuroblasts form large and uniform intercommunicating masses, mostly in direct contact with the surrounding parenchyma. No chains of neuroblasts neither glial tube-like arrangement are evident till the second week of life. Electron microscopy analyses showed that starting from the second postnatal week of life glial processes progressively assemble to form thicker septa, which eventually escheat chains of neuroblasts during the third postnatal week (fig. 1B). In parallel to the morphological changes, several cell surface and extracellular matrix molecules such as PSA-NCAM and tenascin-C, modify their expression in the postnatal SVZ. This temporal pattern coincides with the fully maturation of brain tissue at the end of neurogenesis, thus suggesting that the characteristic arrangement of the adult SVZ glial compartment might mainly have a role in isolating the immature environment and stem cell niche from surrounding mature tissue rather than play a direct structural function as substrate for cell migration.

SVZ ASTROCYETS AND THE NEUROGENIC NICHE

An increasing number of data indicate multiple and complex roles of SVZ glial cells in regulating adult neurogenesis (Alvarez-Buylla and Garcia-Verdugo, 2002 for review). First and quite surprising function of SVZ astrocytes concern their role as stem cell reservoir (Doetsch et al., 1999). Following elimination of both migrating neuroblasts (A cells) and highly proliferating cells (C cells) by using antimitotic drugs, Alvarez-Buylla and colleagues demonstrated that SVZ astrocytes (B cells) were able to divide to generate new C cells that in turn generate A cells. SVZ astrocytes also have neural stem cells properties *in vitro* (Doetsch et al., 1999). The astrocytic identity of the adult neural stem cells has also been demonstrated for the hyppocampus (Seri et al., 2001). Notable, SVZ astrocytes originate from radial glia (Alves et al. 2002; Tramortin et al., 2003; Peretto et al., 2005) which represents the neural stem cells in the developing mammalian neocortex (Malatesta et al., 2000). These data thus indicate that neural stem cells are within the astroglial lineage, but the identification of specific markers to discern between terminally differentiated astrocytes from those with stem cell properties remains one unanswered important question.

In addition to the characterization of the location and properties of the neural stem cells, major efforts are directed to address the mechanisms that regulate stem cells activity. As a result a new concept, the "niche", has been proposed. The niche is considered to be a sub-set of cells and extra-cellular substrates that create the microenvironment capable of indefinitely housing one or more stem cells and that functions to control their self-renewal and expansion *in vivo* (Spradling et al., 2001; Doetsch, 2003). Several data indicate an important role of SVZ astrocytes in creating the neurogenic niche (Lim et al., 2000; Peretto et al., 2002, 2004). Infact, SVZ astrocytes, beside sharing morphological and molecular features with embryonic radial glia (Peretto et al., 1997, 1999: Alves et al., 2002), are implicated in creating molecular barriers (i.e. tenascin-c, Jankovski and Sotelo, 1996), and secrete factors such as the bone morphogenetic proteins (BMPs), and their antagonist noggin (Lim et al., 2000; Coskun et al., 2001; Coskun and Luskin, 2002; Peretto et al., 2002, 2004) which have been demonstrated to play a role in the neurogenic processes. The protein noggin, expressed by ependymal cells of the lateral ventricles (Lim et al., 2000), and SVZ astrocytes (Peretto et al., 2004) can regulate the specifications of newly formed cells toward a neuronal fate by antagonizing the BMPs expressed by the SVZ astrocytes (fig. 1C). Moreover, noggin and several BMPs members have been described all along the SVZ-OB system (Peretto et al., 2002, Peretto et al., 2004). Consistent with the demonstration of stem cells along the entire SVZ-OB system (Gritti et al., 2002), these data suggest that noggin and BMPs could regulate the fate choice all the way from the LV to the OB and rise the possibility of additional roles for these molecules in adult SVZ neurogenesis (i.e. migration and differentiation). Moreover, the specific expression of these morphogens and their receptors in the glial compartment indicates that SVZ astrocytes can regulate this activity through autocrine-paracrine inductive mechanisms. Thus, a subpopulation of astroglial cells of the adult brain can play a dual and fundamental role that is: i) stem cell reservoir and ii) regulator of the stem cell niche. Astrocytes abundance and peculiar organization of their meandering processes, which contact all SVZ cell types, blood vessels, and cerebrospinal fluid, both important sources of neurogenic factors (Hagg, 2005, Sawamoto et al., 2006), suggest that SVZ astrocytes act as sensors cells controlling adult neurogenesis.

MOLECULAR REGULATION OF ADULT SVZ NEUROGENESIS

Based on their ability to influence *in vitro* and/or *in vivo* different steps of adult neurogenesis including progenitor proliferative activity, fate choice,

differentiation and survival, large numbers of others morphogens, growth factors and neurotrasmitters have been indicated as SVZ niche components (see Hagg, 2005 for exhaustive review). In example, FGF-2 and EGF-receptor ligands have been demonstrated to regulate *in vitro* (Palmer et al., 1995; Taupin et al., 2000) the proliferative activity of neural stem cells and seem to be important also *in vivo* (Kuhn et al., 1997; Wagner et al., 1999). Intraventricular infusion of the vascular endothelial growth factor (VEGF) and of the brain-derived neurotrophic factor (BDNF) enhance progenitor proliferation (Benraiss et al., 2001; Pencea et al., 2001; Jin et al., 2002; Zhu et al., 2003), and neural stem cell derived factors also appears essential for the regulation of proliferation in an autocrine fashion (60).

Molecular factors controlling later steps of neurogenesis are currently less clarified. However, PSA-NCAM (Ono et al., 1994), members of the ephrin-B family (Conover et al., 2000), the choroids plexus and septum secreted Slit1 and 2 (Wu et al., 1999; Nguyen-Ba-Charvet et al., 2004), integrin family members (Jacques et al., 1998), reelin (Hack et al., 2002) and Tenascin-R (Saghatelyan et al., 2004) are molecules essential for the migratory process of SVZ neuroblasts. Recently, it has been proposed that directional beating of ependymal cilia, required for normal cerebrospinal fluid flow, determine concentration gradients of cerebrospinal, chemorepulsive, molecules in the SVZ which contribute important vectorial information for guidance of neuroblasts toward the OB (Sawamoto et al., 2006).

Many, if not all, of the stimuli that contribute to such processes have the capacity to signal to the nucleus and influence gene expression. One intracellular molecule that is a potential key target is the cAMP Responsive Element Binding protein (CREB). CREB-mediated gene expression is necessary for survival of multiple neuronal subtypes (Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999; Monti et al., 2002; Shalizi et al., 2003) and it is involved in differentiation, synaptic plasticity and memory (Lonze and Ginty, 2002). Moreover, recent studies have demonstrated transient CREB phosphorylation in neuroblasts of the adult hyppocampal dentate gyrus (Nakagawa et al., 2002a), where this transcription factor regulates several steps of the neurogenic process, including proliferation, differentiation and survival (Nakagawa et al., 2002a; Nakagawa et al., 2002b; Fujioka et al., 2004). In the SVZ-OB system we have recently demonstrated that while CREB is expressed by the SVZ-derived neuroblasts throughout the neurogenic process, its activation through phosphorylation correlates with defined stages of neuronal differentiation (fig. 1C). *In vitro*, inhibition of CREB reduces the morphological maturation of SVZ-derived neuroblasts and experimentally

induced loss of functional connections from the periphery to the OB results in downregulation of CREB phosphorylation in neuroblasts, which is accompanied by reduced radial migration and survival. Moreover, transgenic mice lacking CREB, in a null CREM genetic background, show reduced survival of newborn neurons in the OB. These overall data suggest a novel mechanism by which adult neurogenesis in the OB is regulated by CREB, that supports maturation and survival of SVZ neuronal precursors. Differentiation and survival of new neurons in the OB appear strictly correlated. Infact, most of the SVZ neuroblasts undergo a process of selection (cell death) before, or immediately after maturation has occurred (Petreanu and Alvarez-Buylla, 2002). Interestingly, this process of selection/recruitment of new neurons in the OB is highly regulated by the peripheral olfactory input (Cummings et al., 1997; Rochefort et al., 2002; Petreanu and Alvarez-Buylla, 2002). Odor enrichment or deprivation of the peripheral sensory input modulate survival and probably also radial migration of newly generated cells (Frazier-Cierpial and Brunjes, 1989; Cummings and Brunjes, 1997; Cummings et al., 1997; Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002; Saghatelyan et al., 2004; Giachino et al., 2005), but the molecular mechanism involved is not known. Modifications of the molecular microenvironment and in particular of growth factor levels likely mediate these effects.

FUNCTION OF ADULT OB NEUROGENESIS

The functional meaning of adult neurogenesis in the SVZ-OB system remains quite unclear and highly debated (Bruel-Jungerman et al., 2004; Meshi et al., 2006). Interestingly, besides the demonstration of functional integration of the newly generated cells in the OB pre-existing circuits (Abrous et al., 2005 for review), a recent paper suggested a specific role for the newly generated SVZ cells (Magavi et al., 2005). Although these cells represent a very small percentage of the total OB interneurons, the authors demonstrated that newly formed granule neurons respond to novel odors in larger numbers than did pre-existing granule cells. Moreover, they showed that familiarization with different set of odors increases the response of the newly formed cells, while depresses that of the overall mature granule cells population. Thus, these data indicate different roles for newly-generated and pre-existing OB interneurons, and similarly to the granule cells of hippocampus, an involvement of OB adult generated neurons in mechanisms of neuronal plasticity and learning.

CONCLUSIONS

The study of adult neurogenesis has the main goal to understand the mechanisms allowing integration of new neurons in the mature CNS. This means to investigate a range of phenomena related to crucial neurobiological themes such as the regulation of the stem cell compartments in their specific locations, the proliferation and migration of progenitor cells, the specification of neuronal and glial precursors and their differentiation and integration into different CNS regions. In this contest, the olfactory system provides a unique model in which, the anatomical substrate and the cellular and molecular controls of mechanisms leading the adult progenitor stem-like cells toward functional integrated neurons in the adult CNS, have began to be delineated. Advances in our understanding of these essential ingredients will provide the basis to elucidate the function of adult neurogenesis and it is critical to develop potential therapeutic strategies for CNS diseases, involving activation and guidance of endogenous progenitors towards specific targets and phenotypes.

ABSTRACT

In the last decade, it has been clearly demonstrated that restricted areas of the adult mammalian brain, namely, the subventricular zone (SVZ) and the dentate gyrus of the hippocampus, are neurogenic under physiological condition. Adult neurogenesis in these areas is attributable to the persistence of multipotent progenitor stem-like cells in the mature brain parenchyma. The olfactory bulb (OB) is the brain region most strikingly enriched throughout life by new neurons, whose precursors are generated by the stem cell compartment residing into the forebrain SVZ and then undergo long distance cell migration. Here we describe the structural organization and mechanisms allowing adult neurogenesis in the SVZ-OB system.

RIASSUNTO

Negli ultimi dieci anni è stato chiaramente dimostrato che alcune aree del cervello dei mammiferi adulti, specificamente la zona subventricolare (SVZ) e il giro dentato dell'ippocampo, sono neurogeniche in condizioni fisiologiche. La neurogenesi di queste zone nell'adulto è da attribuirsi alla presenza di cellule staminali multipotenziali nel parenchima del cervello maturo. Il bulbo olfattivo (OB) è la regione del cervello maggiormente arricchita durante la vita da neuroni di nuova formazione, i cui precursori vengono generati da cellule staminali presenti nella zona subventricolare del cervello anteriore. Qui descriviamo l'organizzazione strutturale ed i meccanismi che permettono la neurogenesi in età adulta nel sistema SVZ-OB.

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Multi-disciplinary Prospecting for Navel Orangeworm Attractants

INTRODUCTION

The Navel Orangeworm, *Amyelois transitella*, is the most serious insect pest of almond and pistachios in California and a commercial pest of a number of other crops, including but not limited to walnuts and figs. The navel orangeworm is primarily controlled with organophosphate (OP) and pyrethroid insecticides, but alternative methods of control are sorely needed given the regulations regarding applications of OP and secondary pest problems caused by pyrethroids. Sex pheromones and other semiochemicals are invaluable tools for monitoring and control of insect pest populations. When these chemicals, produced by insects (for their own communication), are accurately identified and synthesized, they may be employed in IPM strategies to monitor populations and determine treatment timing or to reduce populations by mass trapping, attract-and-kill, or mating disruption. A number of economically important lepidopteran species have been successfully controlled using synthetic sex pheromone in mating disruption. This technique has many advantages over conventional insecticides as pheromones are non-toxic chemicals and it is highly unlikely that resistance can be developed. Non-toxicity is not only desired because it leaves no residues, but also there is no effect on natural enemies that keep populations of secondary

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species under control. For the successful application of pheromones in mating disruption, however, it is essential that the synthetic and natural sex pheromone be identical, i.e., the chemicals emitted by female to lure males must be accurately identified. Minimal modifications in the chemical structures of pheromones render them completely inactive. Largely, the pheromone systems of moths contain multiple constituents in a precisely defined ratio. Despite the tremendous effort by leading scientists in the field of chemical ecology over two decades, only one single constituent (Z11Z13-16Ald) from the navel orangeworm pheromone system (Coffelt et al., 1979) was known until recently. We have taken a molecular-based strategy to study chemical communication in this species and to identify a complete pheromone system. In our approach, potential pheromone constituents and attractants are screened with proteins involved in the reception of pheromones. The reception of pheromones and other semiochemicals in insect antennae is initiated by binding to pheromone-binding proteins (PBPs) or odorant-binding proteins (OBPs) that transport the hydrophobic (water insoluble) pheromones through the aqueous environment inside the antennae towards the pheromone receptors (Leal, 2005; Leal, 2005; Leal, 2003; Leal et al., 2005). We have isolated odorant- and pheromone-binding proteins from the navel orangeworm, obtained amino acid sequences of the isolated OBPs and PBPs, cloned the cDNAs (genes) encoding these OBPs and PBPs, developed expression systems for the navel orangeworm OBPs and PBPs, and produced, characterized, and purified recombinant OBPs and PBPs. With a recently developed binding assay, we began screening attractants that led to the discovery a multi-component sex pheromone system which is now being tested in the field and should become commercially available in the near future.

RESULTS

We have generated a large sample of recombinant olfactory proteins from the navel orangeworm utilizing a previously developed expression protocol. We have now studied the secondary structure of the recombinant AtraPBP1 by circular dichroism (CD). As indicated by a maximum at 193 nm and two minima at 208 and 224 nm (fig. 1), AtraPBP1 is a helix-rich protein, a structure previously observed for the pheromone-binding protein from the silkworm moth, *Bombyx mori* (Horst et al., 2001; Lautenschlager et al., 2005; Sandler et al., 2000). Also, AtraPBP1 undergoes a pH-dependent conformational change, as indicated by the change in the second minimum (224 nm) at pH 5 (fig. 1).

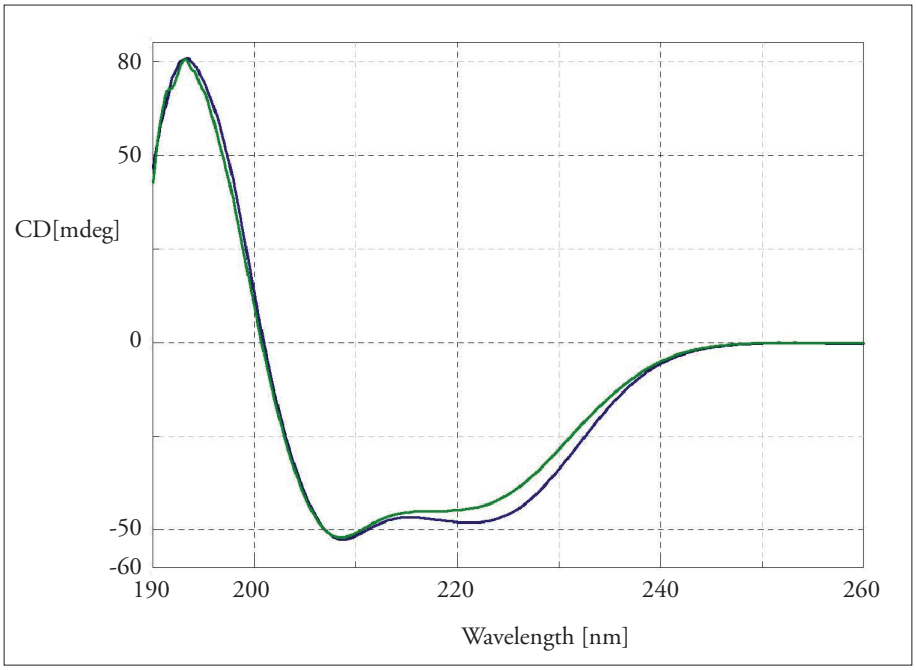

Fig. 1 *CD spectra of AtraPBP1 at pH 7 (bottom) and pH 5 (upper trace)*

This pH-dependent conformational change is characteristic of moth PBPs and it is reflected in one of the two minima in the CD spectra because the C-terminus of the protein folds into a α-helix, whereas the helix in the N-terminus unwinds (Damberger et al., 2000; Horst et al., 2001; Lautenschlager et al., 2005; Wojtasek and Leal, 1999). pH-Titration by intrinsic fluorescence also suggest that AtraPBP1 has two conformations one at the sensillar lymph pH (high pH) and the other at low pH (fig. 2).

We have developed a new binding assay based on the separation of bound and free ligands by a centrifugal device. After incubation of a test compound with a PBP, the free ligand is removed by filtration, whereas the ligand bound to the protein is extracted with organic solvent and analyzed by GC and GC-MS (Leal et al., 2005). Using this novel binding assay, we have observed that binding of the major pheromone constituent, Z11Z13-16Ald to AtraPBP1 is pH dependent, with high binding affinity at high pH and no binding at low pH. These experiments confirmed that this male-specific olfactory protein binds a constituent of the navel orangeworm pheromone and is, therefore, a pheromone-binding protein.

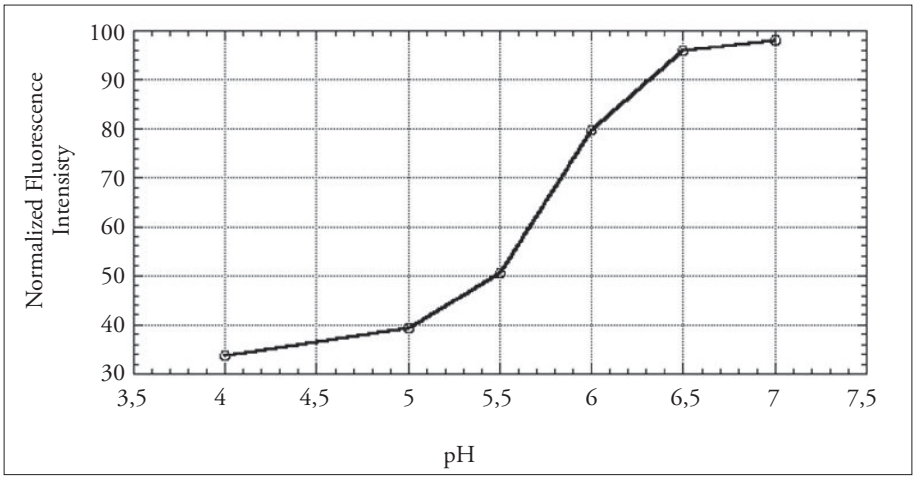

Fig. 2 *Effect of pH on the intrinsic fluorescence of AtraPBP1*

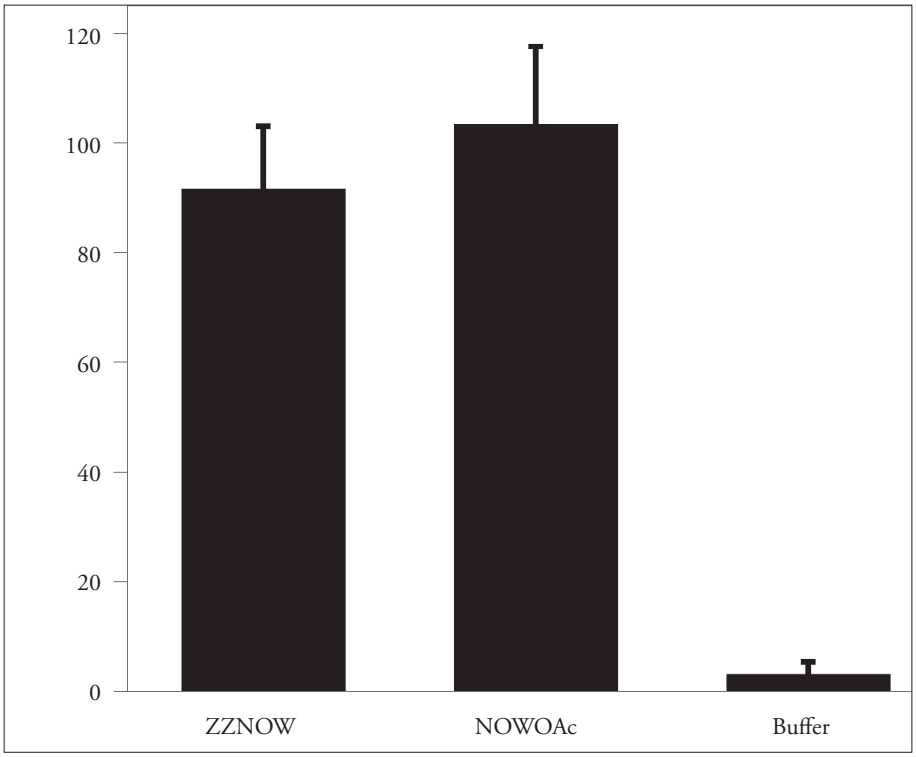

Fig. 3 *Binding of (Z,Z)-11,13-hexadecadienal [ZZNOW] and (Z,Z)-11,13-hexadecadienyl acetate [NOWOAc] to AtraPBP1 at pH 7. Only traces of these ligands were detected when protein was replaced by buffer (control)*

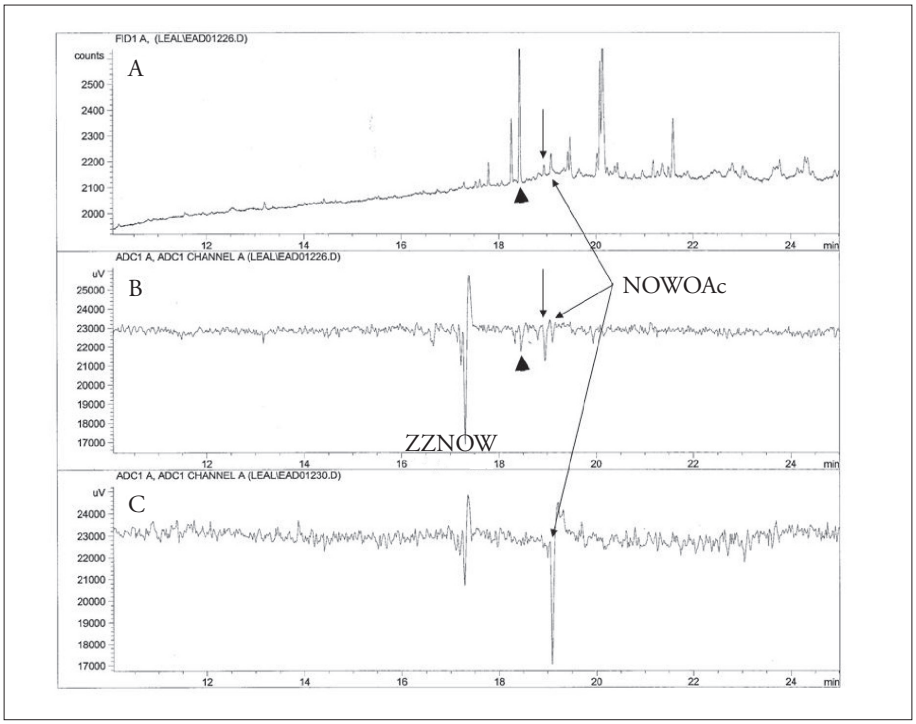

Fig. 4 *Gas chromatography (A) with simultaneous electroantennographic (B) recording (GC-EAD) obtained with gland extract from the navel orangeworm after separation on a silica gel column (3% fraction) and using male antenna as the detector. The peak of NOWOAc in the gland extract (A), and EAD (B) was confirmed with a mixture of a sample of NOWOAc (C) and ZZNOW for reference*

Having validated AtraPBP1 as a pheromone-binding protein, we used this "molecular target" to fish out other ligands, i.e., other potential attractants for the navel orangeworm. We observed that AtraPBP1 binds (Z,Z)-11,13-hexadecadienyl acetate (NOWOAc, in short) with the same apparent affinity as that observed for (Z,Z)-11,13-hexadecadienal (ZZNOW, in short) (fig. 3).

The molecular evidence supporting that (Z,Z)-11,13-hexadecadienyl acetate binds to AtraPBP1 prompted us to investigate the occurrence of this semiochemical in the female pheromone gland. To determine the optimum time for extraction of pheromone glands, we studied initially the sexual behavior of the navel orangeworm (Parra-Pedrazzoli and Leal, 2006). We then extracted with hexane pheromone glands excised from virgin females at the time of the peak of pheromone production and calling behavior. Crude extracts were analyzed by single sensillum recordings (SSR), in which a response from a single phe-

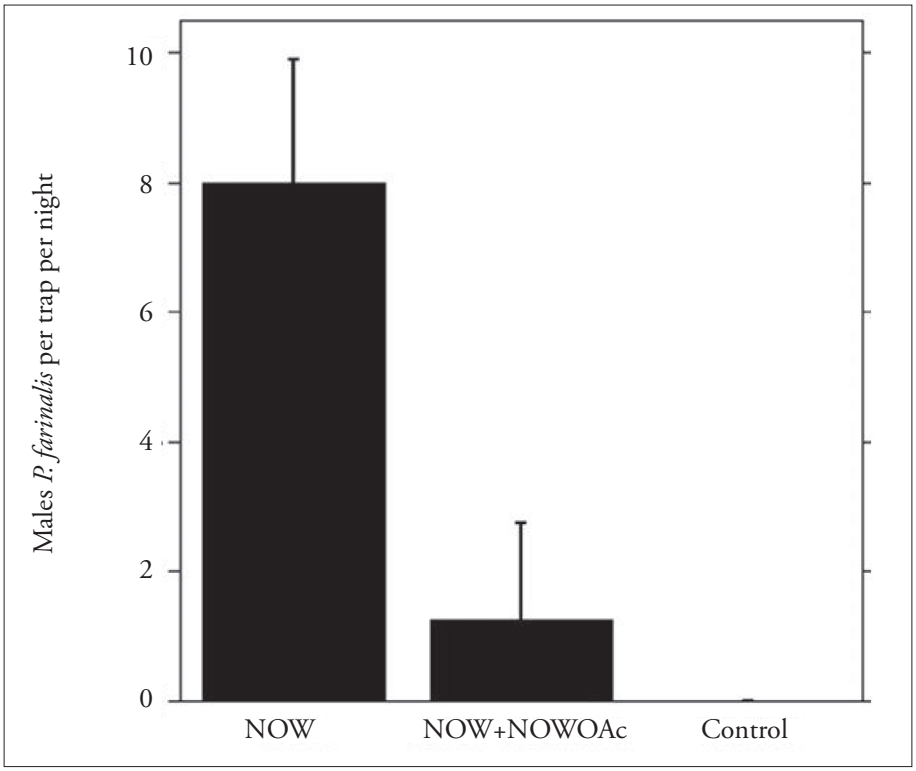

Fig. 5 *Effect of (Z,Z)-11,13-hexadecadienyl acetate [NOWOAc] on the capture of males P. farinalis with the previously known constituent of the navel orangeworm sex pheromone, (Z,Z)-11,13-hexadecadienal [NOW]*

romone detector on the antennae is recorded. SSR indicated that the pheromone gland extracts contained multiple components as multiple neurons were activated. Flash chromatography on the crude extract generated one fraction (3%) in which esters and acetates are normally eluted. This 3% fraction showed also electrophysiological activity by SSR. When analyzed by GC-EAD, a small peak corresponding to (Z,Z)-11,13-hexadecadienyl acetate (NOWOAc) was detected (fig. 4). The retention time was confirmed by an authentic sample of NOWOAc both in a non-polar (fig. 4C) and a polar column (data not shown). Also, GC-MS and GC-FTIR data from the peak confirmed that the navel orangeworm produces (Z,Z)-11,13-hexadecadienyl acetate.

Preliminary field and indoor bioassays suggested that NOWOAc is neither an attractant nor an inhibitor for the navel orangeworm. However, field tests clearly indicated that NOWOAc is a behavioral antagonist for *P. farinalis*.

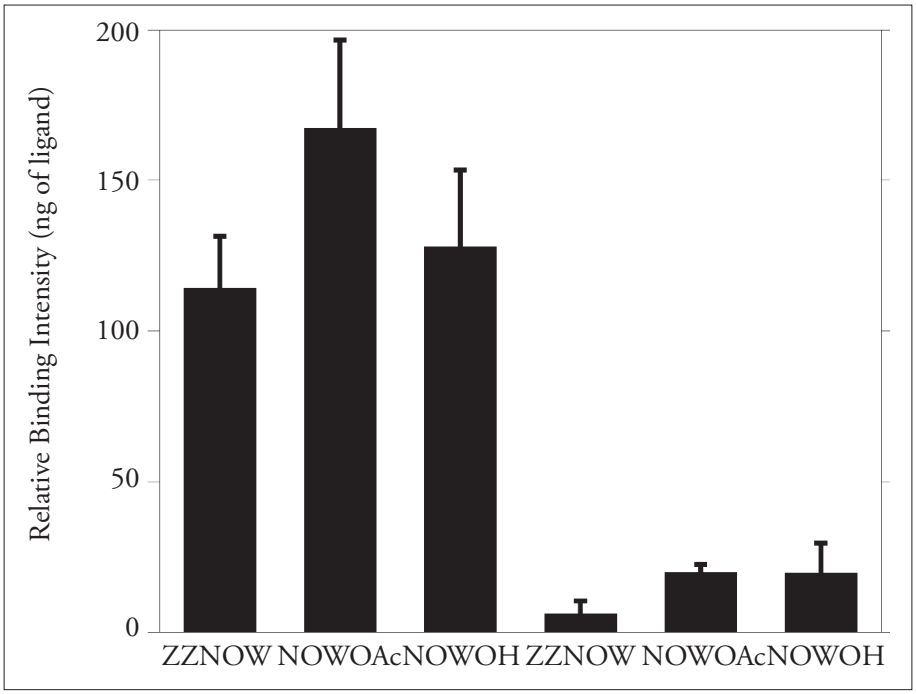

Fig. 6 *Competitive binding data. The right side of the panel shows the tests with buffer only (controls)*

Traps baited with the major constituent of the navel orangeworm sex pheromone (ZZNOW) caught males of *P. farinalis*, albeit in small numbers, but no males of the navel orangeworm. Interestingly, catches of male *P. farinalis* by traps baited with lures containing both ZZNOW and NOWOAc decreases significantly (fig. 5). These data indicate that NOWAc is a behavioral antagonist, which may be produced by the navel orangeworm to avoid attracting males of the wrong species.

Similar fractions obtained from different gland extracts showed a consistent profile as the one shown in figure 4. A strong EAD-peak for (Z,Z)-11,13-hexadecadienal (ZZNOW) was detected because small amounts of this compound eluted in the ester fraction (3%). Two other EAD-active peaks appear between ZZNOW and NOWAc, one indicated by an arrow and the other by an arrowhead (fig. 4). These compounds were identified as ethyl (Z,Z)-hexadecadienoate and ethyl palmitate, respectively.

An additional "molecular hint" was derived from our studies on the molecular basis of pheromone reception in the navel orangeworm. While screening

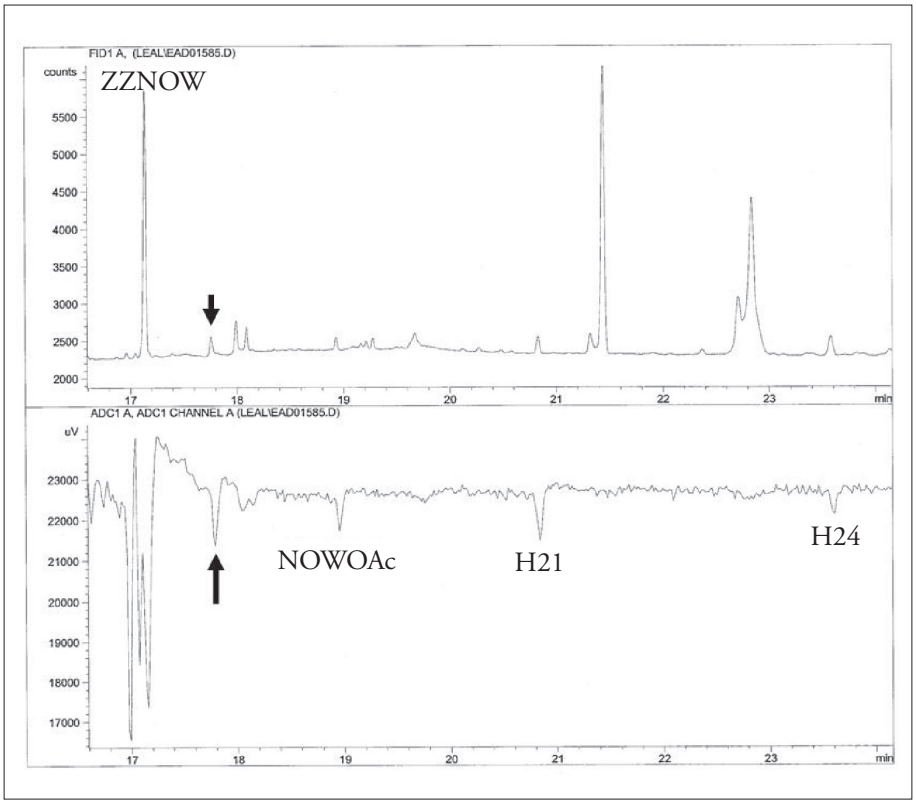

Fig. 7 *GC-EAD recording from a crude gland extract. In addition to ZZNOW, four other EAD active peaks are highlighted: NOWOAc, two novel pentaenes, H21 and H24, and (Z,Z)-11,13-hexadecadienol [NOWOH] indicated by an arrow*

for other potential ligands for AtraPBP1, we observed that (Z,Z)-11,13-hexadecadienol (NOWOH, in short) binds to our "molecular target" suggesting a potential role in olfaction. As demonstrated in competitive binding assays, in which AtraPBP1 was exposed to the three ligands at the same time, AtraPBP1 showed slightly higher preference for the behavioral antagonist (NOWOAc), whereas both ZZNOW and NOWOH showed apparently the same binding affinity (fig. 6). Indeed, this compound is also produced in the pheromone gland, as indicated by the analysis of a crude hexane extract of virgin female glands excised at the time of the peak of pheromone production (fig. 7).

Other EAD-active compounds produced by pheromone gland, in addition to ZZNOW, NOWOAc, and (Z,Z)-11,13-hexadecadienal (fig. 7), are two novel pentaenes, H21 and H24. These compounds have been fully identified as (Z,Z,Z,Z,Z)-3,6,9,12,15-tricosapentaene and (Z,Z,Z,Z,Z)-3,6,9,12,15-

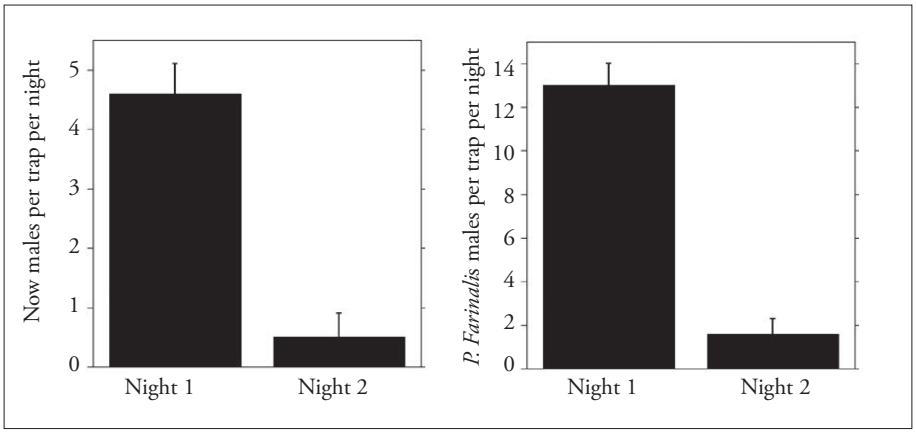

Fig. 8 *Capture data for the navel orangeworm (left) and P. farinalis (right) indicating a dramatic decrease in activity after one day exposure in the field. Control (empty) traps caught no males and were omitted in the figure*

pentacosapentaene (Leal et al., 2005). Other minor constituents identified by GC-EAD are (Z)-11-hexadecenal and (Z)-13-hexadecenal.

All compounds identified as sex pheromone attractants and the behavioral antagonist were synthesized by Bedoukian Research Inc (Leal et al., 2005). Due to possible chemical instability of some of the pheromone constituents, synthetic lures were formulated and evaluated in Davis where populations of the navel orangeworm are very low. Clearly, the new synthetic lure is active (fig. 8), but long-lasting formulations are still under development. When the synthetic pheromone was formulated in rubber septa, trap captures during the first night that the traps were deployed were high (given the low population), but the captures decreased dramatically on the second night in the field (fig. 8). This was observed not only with NOW, but also with *P. farinalis*, which has a higher population in the location where the tested were conducted.

In general, pheromones formulated in rubber septa do not last long in the field, but the lures are active for at least 3-5 days. The rapid decrease in activity of the NOW pheromone formulated in rubber septa suggests additional problem(s), which may be derived at least in part from exposure of the pheromone to sun light. Thus, for monitoring populations of the navel orangeworm a long-lasting formulation is still needed. However, this may not be the case with applications of the new synthetic pheromone when used for mating disruption and applied using a 'puffer'. Here, the pheromone mixture is canned and thus protected from sun light. A puffer may be programmed

to release intermittent sprays of pheromone mixture (in a precise ratio) in the dark during the time of flight and mating activity of the navel orangeworm.

In an attempt to develop additional attractants, we have screened a large number of plant volatiles, floral compounds, and other semiochemicals by both electroantennogram (EAG) and gas chromatography with electroantennographic detection (GC-EAD) using male and female antennae. We identified two compounds, which generate electroantennographic detection by male and female antennae, a potential unisex attractant. In addition, we have identified a number of compounds, which generate strong EAD activity in female, but not male antennae. These compounds are potential ovipostion attractants.

ACKNOWLEDGMENTS

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ABSTRACT

Employing a multi-disciplinary approach, including but not limited to a non-conventional protein-based screening technique, sensory physiology, and state-of-the art analytical instrumentation, we discovered eight additional constituents of the pheromone system of the navel orangeworm, a major agricultural pest in California. We isolated, cloned, expressed, and characterized odorant- and pheromone-binding proteins (PBPs). Then we employed recombinant PBPs to screen for potential attractants. Concomitantly, we studied the sexual behavior of the navel orangeworm, extracted pheromone glands from calling females, analyzed gland extracts by single sensillum recording and gas chromatography with electroantennographic detection, isolated and identified the electrophysiologically-active compounds. Field tests indicated that one of the newly identified compounds is a behavioral antagonist produced by females of the navel orangeworm to repel males of Pyralis farinalis. The other new constituents are sex attractants. Traps baited with the new sex pheromone system, formulated in rubber septa, caught significant numbers of males of the navel orangeworm, even in areas with low populations. Long-lasting formulations for commercial applications in monitoring are under development. The new synthetic pheromone has potential application in a mating disruption strategy for controlling populations of the navel orangeworm. We are exploring the development of additional attractants, particularly those targeting gravid females.

RIASSUNTO

Mediante l'uso di un approccio multidisciplinario, comprendente tecniche di screening di proteine non convenzionali, fisiologia sensoriale ed attrezzature analitiche di ultima generazione, abbiamo identificato otto nuovi componenti della miscela feromonica dell'insetto dell'arancio, una delle specie piu' dannose all'agricoltura in California. Abbiamo isolato, clonato, espresso e caratterizzato proteine leganti odori e feromoni. Successivamente abbiamo utilizzato le proteine leganti i feromoni per cercare possibili sostanze attrattive. Allo stesso tempo abbiamo studiato il comportamento sessuale dell'insetto, abbiamo estratto le ghiandole che producono i feromoni nella femmina, analizzato il loro contenuto mediante gascromatografia ed elettroantennogrammi ed infine identificato i componenti attivi. Prove in campo hanno indicato che uno dei nuovi composti identificati e' un antagonista che allontana i maschi di *Pyralis farinalis*. Gli altri nuovi composti sono attrattivi sessuali. Trappole contenenti la nuova miscela feromonica hanno catturato un gran numero di maschi dell'insetto dell'arancio, anche in zone con basse popolazioni. Formulazioni di lunga durata per applicazioni commerciali sono attualmente in fase di studio. La nuova miscela feromonica sintetica presenta potenziali applicazioni nel controllo di popolazioni di questo insetto mediante la tecnica della confusione. Attualmente stiamo anche studiando lo sviluppo di altre sostanze attrattive, in particolare verso le femmine gravide.

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Krishna C. Persaud*

Design and performance of electronic noses

1. introduction

The remarkable capabilities of the biological chemosensory systems in detection, recognition, discrimination and quantitation of intensity of complex mixtures of chemicals, has stimulated the development of electronic analogues. The human sense of smell is influenced by many factors such as age, gender, state of health and mood. The response of human subjects to different odours and odour concentrations is highly subjective as the human sense of smell can vary dramatically from person to person. An instrument that could perform simple odour discrimination and provide measurement of odour intensity, without subjective influences, would be very useful in modern industry.

2. key concepts

The survival of animal life in complex, changing environments requires the use of sophisticated sensory systems to detect, classify and interpret patterns of input stimulation. It is now known that the receptor cells and the receptor proteins they express generally respond to more than one compound. Ensembles of these neurons encode the detailed information about the chemical environment on which animals base their olfactory behaviours. Some of the fundamental principles of pattern classification that seem to be common from biological systems to artificial systems would appear to be 'template matching',

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whereby the pattern to be classified is compared with a set of templates, one for each class, the closest match determining the classification, and 'feature detection systems' in which a number of measurements are taken on the input pattern and the resulting data is combined to reach a decision. These systems may involve either a sequential approach whereby information from the evaluation of some features is used to decide which features to evaluate next, or a parallel approach where information about all features are evaluated at the same time with no weight being placed on any particular feature.

A characteristic of the activity of the nervous system of an animal is that it is persistent throughout the life of the animal, and so is in some sense stable. However, it also appears to have a stochastic nature that is essential - a nervous system that is stereotyped would not exhibit adaptive behavior in a changing environment. Animals have goal driven behavior that works reliably in an unpredictable world. The methods of pattern classification mentioned can be elaborated so that stochastic behaviour is achieved, and many models that may be applicable to the sensory systems of animals have developed. Evidence from chemistry, (Beets, 1978), olfactory psychophysics and structure-activity relationships of odorants, (Boelens, 1974), together with the examination of 'specific anosmias' in the human population, all support the definition of selectivity and specificity of putative olfactory receptors initiated by Amoore (Amoore, 1962b; Amoore, 1962a; Amoore, 1967) and confirmed by recent developments in olfactory neurobiology and molecular genetics (Buck, 1997a; Buck, 1997b; Chess et al., 1992; Mombaerts et al., 1996). The ideas that several classes of olfactory receptors exist, selective to chemical species on the basis of molecular size, shape and charge, also pointed to individual olfactory receptors being rather broad in their selectivity to molecules within certain classes. The important molecular parameters of an odorant determining the olfactory response would include the adsorption and desorption energies of the molecule from air to a receptor interface, partition coefficients, electron donor/acceptor interactions depending on the polarisability of the molecule, and its molecular size and shape. Olfactory code – highly distributed spatial component

In the olfactory system there is a tendency for receptors with similar characteristics to be segregated at discrete locations. The property of broad tuning might be an advantageous adaptation for a generalist chemosensory system: a system of sensors which respond to a high proportion of possible stimuli (between 20 and 40%) provides substantially greater coding capacity than a system with more selective sensors.

The following main problem areas could be early identified in the design of a 'model' olfactory system.

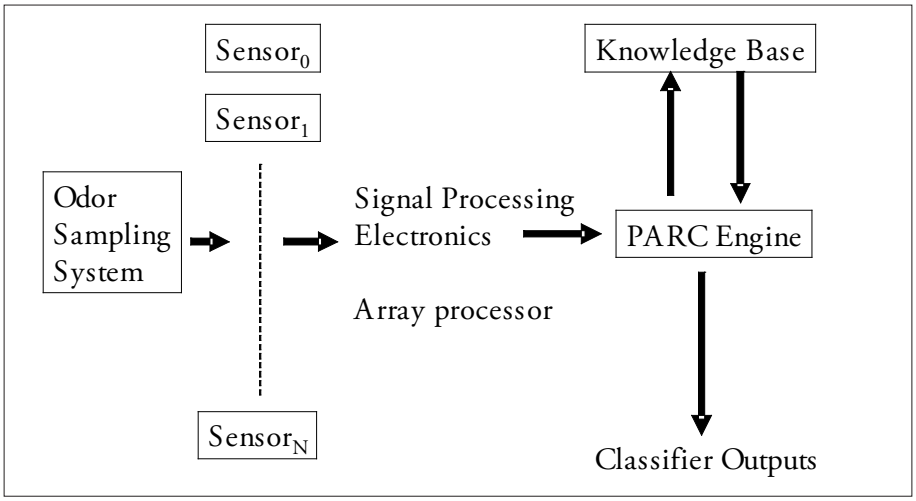

Fig. 1 *Elements of an "Electronic Nose" system. Odour is sampled and presented to individual elements of a sensor array simultaneously. Signals transduced from the sensors are converted to digital format, normalised and presented to a pattern recognition engine (PARC) that outputs some parameter that is a determining characteristic of the incoming odour. This may be discrimination from other known odour classes, or a mapping to some sensory quality determined by a human panel*

(i) The selection of an effective set of parameters for the description of the patterns in question. Where an odour is concerned, the problems to be addressed are which characteristics of the molecule can be measured, and how to encode the resulting pattern.
(ii) The selection of the decision procedure – how is the receptor output to be categorised?
(iii) The selection of a procedure for processing the parameters chosen to represent the pattern so as to optimise the parameter values. This increases the resolution of the system so that certain representative pattern classes may be differentiated from others.
(iv) The selection of 'hardware' required for the simulation of the transduction, coding and pattern recognition activities of the olfactory system.

3. ARRAY BASED ODOUR SENSORS

The utilisation of cross-sensitivities between sensor elements in a chemical sensor array enhances the discriminatory power of a small sensor array. The

responses of the individual sensors, each possessing a slightly different response towards the sample odours, when combined by suitable mathematical methods, can provide enough information to discriminate between sample odours. Such systems have been given the terminology 'electronic nose' and consist of an array of chemical sensors possessing broad specificity, coupled to electronics and software that allow feature extraction - extraction of salient data for further analysis, together with pattern recognition - identification of sample odour. Software techniques and material science are important aspects of the development of the system. Advancement in software signal processing techniques, coupled with pattern recognition, enable optimum usage of sensor responses. The specificity and sensitivity of existing chemical sensors are constantly being developed, as well as new materials.

Typically an electronic nose consists of three elements: a sensor array which is exposed to the volatiles, conversion of the sensor signals to a readable format, and software analysis of the data to produce characteristic outputs related to the odour encountered (fig. 1). The output from the sensor array may be interpreted via a variety of methods such as pattern recognition algorithms, principal component analysis, discriminant function analysis, cluster analysis and artificial neural networks to discriminate between samples.

3.1 *Sensor Technology*

A large number of sensor technologies are now available that are applicable to construction of sensor arrays for "Electronic Nose" applications. Figure 2 illustrates three common sensor types - quartz crystal microbalance, metal oxide and conducting polymer. The sensors must meet key design parameters for the system. These include sensitivity, speed of operation, cost, size, manufacturability, the ability to operate in diverse environments, and immunity to poisoning. The sensors must adsorb large numbers of molecules of a particular species to produce a measurable effect on the sensor that can be transduced into a signal.

3.1.1 Metal Oxide Sensors

Many researchers have chosen commercially available metal oxide sensors such as Taguchi Gas Sensors (TGS) (Figaro Inc., Japan) or Capteur Sensors (Capteur, UK) as the core sensing element in their investigation of array based odour detectors. These devices consist of an electrically heated ceramic

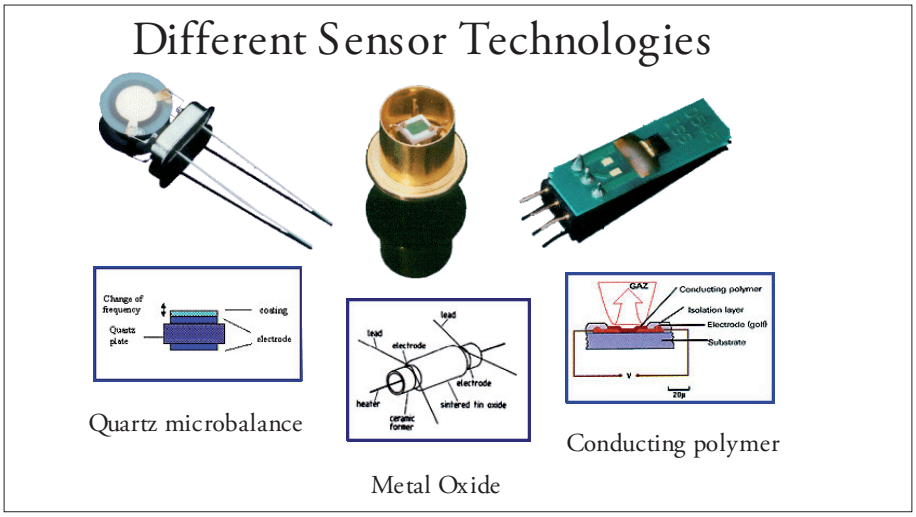

Fig. 2 *Common Sensor Technologies used in Electronic Noses – (a) quartz crystal microbalance, (b) metal oxide sensor, (c) conducting polymer*

pellet onto which a thin porous film of SnO_2 doped with various precious metals has been deposited. The doped SnO_2 behaves as an n-type semiconductor and the chemisorption of oxygen at the surface results in the removal of electrons from the conduction band. Gases interact with the surface adsorbed oxygen and thereby affect the conductivity of the SnO_2 film. The devices are run at elevated temperatures (typically 300 - 400°C) to achieve rapid response/recovery times and to avoid interference from water. This results in relatively high power consumption. The response characteristics can be tailored by varying the operating temperature and the doping agent. The physical and chemical mechanisms by which gases can transduce signals are relatively well understood (Kohl, 1989; Kohl, 1991; Kohl, 1996). Sensors have been developed for the detection (down to the ppm level) of a range of target molecules including H_2, CO, NH_3, H_2S, NO_x, SO_x, ethanol and hydrocarbons.

As an alternative to the commercially available metal oxide sensors, several research groups have fabricated thin film SnO_2 arrays using planar microelectronic technology. Potentially these could have a number of advantages including a reduction in size and lower power consumption.

An elegant variation on this device technology is a sensor array with a gradient of temperature across the surface as well as a membrane sputtered on the surface (Menzel and Goschnick, 2000). These arrays are now commercially manufactured and incorporated into “Electronic Nose” devices (Ehrmann, 1998).

3.1.2 Quartz resonator and Surface Acoustic Wave Devices

Quartz resonator gas sensors consist of a piezoelectric quartz crystal oscillator coated with a sensing membrane. Typically a quartz disk is sandwiched between two electrodes. The adsorption of volatile molecules onto the membrane results in a decrease in the resonant frequency due to the increased mass. This frequency shift can be used as the sensor output and the device response can be varied by using different membrane materials (Nanto, 1997).

Surface acoustic wave (SAW) and bulk acoustic wave (BAW) devices consist of interdigitated electrodes fabricated onto a piezoelectric substrate (e.g. quartz) onto which a thin film coating of a selective material is deposited. An applied radio frequency voltage produces a Rayleigh surface acoustic wave (i.e. a surface oscillation). Adsorption of odours onto the coating increases its mass and perturbs the wave leading to a shift in frequency. To compensate for pressure and temperature effects the sample sensor is usually connected to a reference SAW device and the frequency difference is detected. As with quartz resonator devices the coating material determines the selectivity. SAW devices however can be operated at higher frequencies, which it is claimed results in improved sensitivity (Rapp and Reibel, 1996; Yang, Yang, and Wang, 2000). The advantages of SAWs and BAWs include high selectivity, high sensitivity, stability over wide temperature ranges, low response to humidity, and good reproducibility. The disadvantage is the complexity in the interface electronics.

3.1.3 Electrochemical Sensors

Electrochemical sensors are widely used for detection of specific chemical analytes. Typically a redox reaction is induced to occur at the anode or cathode of an electrochemical cell when a specific gas is dissolved in the electrolyte. Commercial sensors are made by a variety of companies such as City Technology Ltd, UK. A variety of amperometric or coulometric devices are applicable to sensor arrays for "Electronic Noses" (Stetter, 1995).

3.1.4 MOSFET Sensors

MOSFET's (Metal Oxide Semiconductor Field Effect Transistors) have been used as gas detectors, the vapour producing a shift in the capacitance-volt-

age characteristic. MOSFET sensor behavior can be modified with coatings of zeolite of various pore sizes or by careful attention to a gas-sensing layer in close proximity to the gate of the MOSFET, resulting in a change of capacitance on exposure to the gas. Using arrays, patterns can be generated for a range of solvent volatiles, and chemicals such as ammonia and hydrogen (Gardner, 1998; Stetter et al., 2000). Some "Electronic Nose" companies now commercially use such sensors.

3.1.5 CHEMFETS

A chemical field effect transistor (ChemFET) is a transistor with the gate electrode coated with a selective coating. This coating adsorbs odorant molecules, which changes the conductivity across the transistor's gate. The advantages include high sensitivity (ppm), high selectivity, and ease of integration with other electronics. A potential disadvantage is that the odorant molecules must penetrate the transistor gate, and the devices are not yet available commercially. Variations incorporating conducting polymers as chemical sensing surfaces have been devised (Hatfield, Covington, and Gardner, 2000).

3.1.6 Optical Sensors

A fibre optic sensor for gas sensing is a conventional optical fibre typically coated with a coating, which interacts with the odorant molecules. The coating may be a fluorescent dye. An optical pulse is applied to the sensor and is adsorbed by the coating. The interaction of the odorant molecules and fluorescent dyes produces a frequency shift in the returned fluorescent signal. The returned signal is then analysed to determine the properties of the odorant molecules.

Porphyrins and other materials may also be used as the active adsorbent material in optical sensing (Di Natale et al., 2000). More recently, microscopic polymer beads impregnated with a fluorescent dye have been incorporated into the ends of optical fibres and these have proven to be extremely sensitive to vapours, and "optical electronic noses" have been developed. Imaging optical fibres in conjunction with two-dimensional detectors such as CCD cameras have been used to fabricate array sensors. These sensors contain spatially separated photopolymers containing analyte-sensitive fluorescent indicators

on an imaging fibre tip. Spatial resolution of the indicators is maintained through the imaging fibre array and projected onto a CCD detector (Dickinson et al., 1998; Dickinson et al., 1999; Dickinson et al., 1996; Walt et al.; Walt et al., 1995).

3.1.7 Mass spectrometry based devices

"Virtual" chemical sensors based on a mass spectrometric approach have been developed for multicomponent analysis of organic vapours. The sensing principle is based on the injection of a complex sample headspace into a mass spectrometer, creating a mass spectrometric pattern of the unresolved gaseous mixtures. After selecting particular fragment ions, the resulting reduced mass spectrum of the sample is treated with pattern recognition (software) typically based on principal components analysis or neural networks (Dittmann et al., 2000; Dittmann, Horner, and Nitz, 1999; Dittmann and Nitz, 2000; Dittmann, Nitz, and Horner, 1998). This methodology has been commercialised by a number of companies such as Agilent Inc., HKR Sensor Systeme (Germany), and Alpha MOS, France.

3.1.8 Carbon black/ Polymer Composite materials

Cyrano Sciences Inc. (USA) have commercialised portable instruments based on 32 carbon black/polymer composite materials. The carbon black forms the conducting phase of the sensor and is dispersed into an insulating organic polymer. When the polymer comes into contact with an organic vapour it swells causing a change in the electrical resistance of the sensor. An electrical potential is applied across each sensor so that the resistances may be recorded. The use of different polymers such as those used in gas chromatography columns, gives the sensors a range of sensitivities to a variety of chemicals (Lewis and Freund, 1996; Lewis and Severin, 1997).

3.1.9 Organic Materials

Much research has been carried out into sensors based on metal-substituted phthalocyanines, each acting as a simple chemiresistor. Their applications

have been limited to detection and measurement of gases such as NO_2 and H_2S (Cranny and Atkinson, 1992), and they have suffered from poor individual sensor response and reproducibility.

On the other hand, the unique electrical properties of organic conducting polymers, derived from aromatic and heteroaromatic materials has led to a large amount of research and application of these materials in different areas. Since 1979 when Diaz et al (Diaz, Kanazawa, and Gardini, 1979) first prepared polypyrrole as a freestanding film, many thousands of publications have appeared. Conducting polymer gas sensors based on measuring resistance changes in thin film structures have been studied by a number of researchers. Configurations have been used to measure the shifts in the work function caused by the adsorption of a range of organic volatiles. The response of the polypyrrole film (i.e. the magnitude and the sign of the work function shift) is determined by the electrochemical deposition conditions and in particular the electrolyte/solvent system used. Measurements have also been made of the change in the optical absorption spectra on exposure to organic vapours. This data together with the work function shifts, suggest a small but reversible charge transfer (either donor or acceptor) when a gas is adsorbed at the polymer surface.

Gas sensors using polypyrrole films deposited as an over layer onto quartz resonator devices have been investigated by Slater and co-workers (Slater and Watt, 1991). In addition to monitoring the mass loading affect of adsorbed volatiles a simultaneous measurement of conductivity changes was made on a separate device. A range of volatiles has been studied including NH_3, methanol, cyclohexane, acetone and H_2S.

Since 1985, Persaud *et al.* (Persaud and Pelosi, 1985) (Persaud et al., 1996b) have concentrated on development of conducting polymers as odour sensing devices, and many materials have been synthesised and characterised for odour transduction. The reasons for choosing conducting polymers as odour sensor elements are as follows.

(a) The sensors show rapid adsorption and desorption kinetics at room temperature.
(b) The sensor elements feature low power consumption (in the order of microwatts) as no heater element is required.
(c) The structure of the polymer can be closely correlated to specificity towards particular classes of chemical compounds.
(d) The sensors are resilient to poisoning by compounds that would normally inactivate some inorganic semiconductor type sensors.

These are reviewed in Persaud (2005).

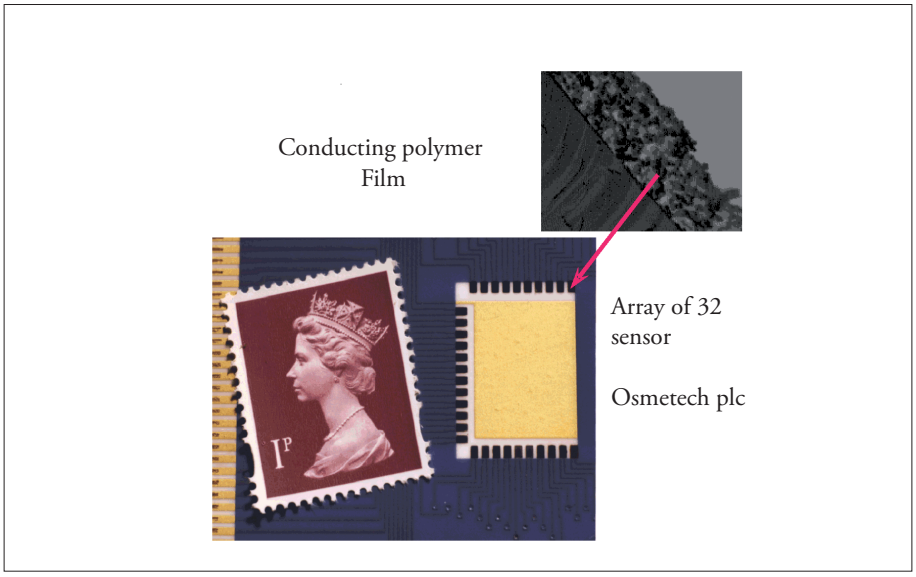

Fig. 3 *(a) Commercial Electronic Nose System – Aromascan A32S (courtesy Osmetech plc). (b) Conducting polymer sensor array (32 sensors) used in the system*

3.1.10 Electronic Nose Hybrid Technology

The manufacturer or the user are not restricted to using only one type of technology, and no one technology is yet generally applicable to generic odour sensing. Hence the use of modular sensor systems, or integrated sensor systems containing different sensor technologies are a growing trend (Kohl, 1997; Stetter and others, 2000).

3.2 *Sensor Output and Data Processing*

Figure 3a shows a commercial instrument – the Aromascan A32S (Osmetech plc) based on a conducting polymer sensor array (fig. 3b). This was launched in 1994, and several hundred units were sold.

No matter which sensor technology is utilised, the raw data response of each sensor in the array to a volatile sample such as shown in figure 4(a) is normalised as shown in figure 4(b) and the resulting pattern is used as descriptor for discrimination between different samples. Sensing systems have been developed where individual elements in an array show broad, and overlapping selectivity's to chemical species, each sensor element responding more selectively to certain groups of chemicals. This approach has the advantage that the array can respond to many thousands of chemical species due to the broad selectivity of the adsorbent surfaces. On the other hand, extremely selective information for discrimination between adsorbed chemical species or mixtures can be obtained by analysis of the cross-sensitivities between sensor elements. The relative responses between sensor elements produce patterns that may be unique 'fingerprints' that may be used as odor descriptors. There are often uncontrolled factors present in measurement data due to effects such as day-to-day variations in ambient temperature and instrument signal drift, which can introduce systematic error into the data.

The aim of many pattern recognition techniques is to identify similarities and regularities present in the data. One method is Cluster Analysis, which attempts to find natural classifications in data. Computers are usually used as an aid in the cluster analysis of data of more than three dimensions (since it is difficult for humans to visualize such vector spaces). The centers of clusters are represented by a set of coordinates, and these are called codebook vectors.

A typical chemometric goal in "Electronic Nose" applications is to find variables to separate known groups, and in particular, to be able to classify

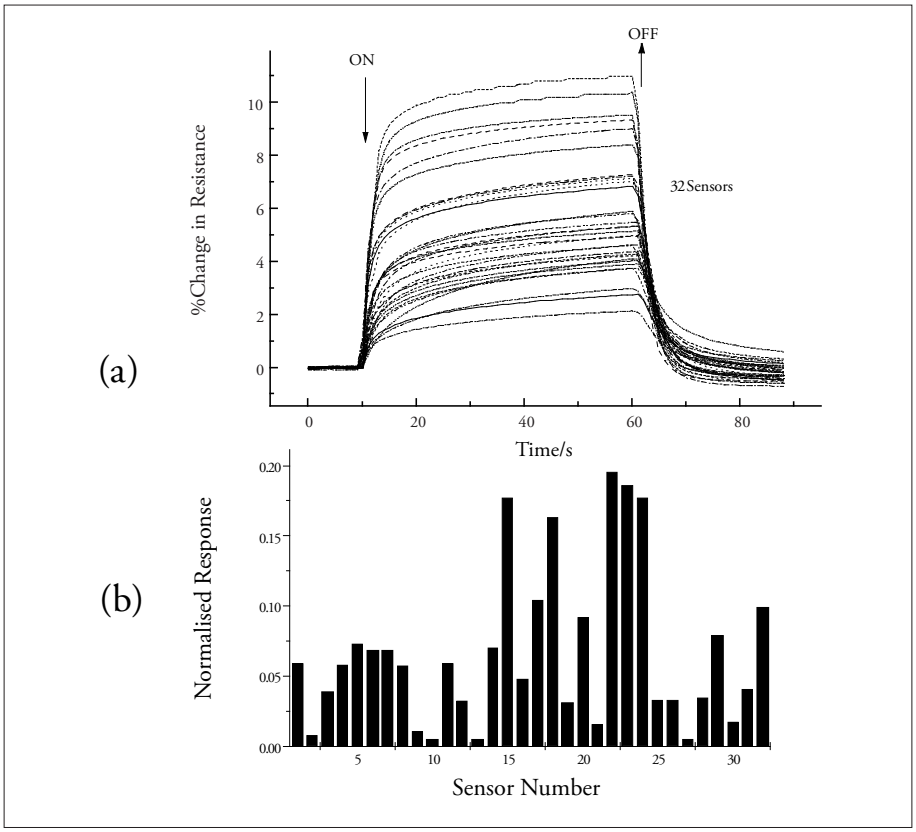

Fig. 4 *(a) Raw data response from a sensor array made from conducting polymers. Each sensor responds to the same concentration of analyte with different sensitivity. By normalising the response across the entire array, a pattern (b) is obtained that is a descriptor of the analyte presented to the sensor array*

a gas or mixture of gases according to various properties. A primary aim of chemometrics is to reduce the number of dimensions used to represent the characteristics of the data set. Various methods are available to accomplish this, either by considering only a subset of the original variables, or by creating a more efficient representative set of new variables. The creation of new variables can be approached in a number of ways; two of these are projection and mapping.

Projection is more common and involves using a weighted linear combination of the original variables to derive a new, more compact data set that contains nearly the same informational content as the original variables. Clearly this involves a certain amount of loss of data, and this is not ideal sin-

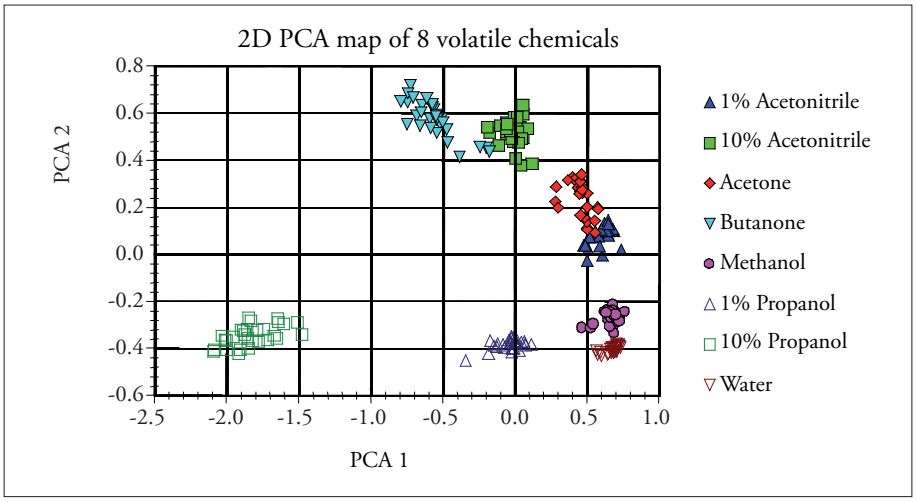

Fig. 5 *A principal component analysis of replicate samples of different analytes. Each point represents a multidimensional pattern from 32 sensors projected onto two dimensions*

ce key features may be discarded, and so techniques have been developed to minimize information loss. A commonly used projection technique is Principal Components Analysis (PCA) (Hotelling, 1933). The purpose of the PCA transformation is to rotate the old co-ordinate system in a direction so that the new system will have most of the relevant information aligned along a few new axes. The majority of the new axes will carry a very small proportion of the total information, and could be disregarded without too much loss. It attempts to maximize the variance information present in data in the minimum number of mutually orthogonal dimensions. Graphically, PCA distorts the axes to conform to axes that contain a maximum of variance information. One way to describe this would be to consider looking at the data from a different direction in space. Algebraically, this can be performed as a simple linear transformation in any number of dimensions (See fig. 5).

Mapping is a similar technique to projection, but the data transformations are non-linear, and attempt to preserve key properties of the data (such as the distances between points), while reducing the number of dimensions. Other chemometric techniques applied include partial least squares (PLS), discriminant analysis (DA), and discriminant factorial analysis (DFA). DFA is a multivariate technique that determines a set of variables which best discriminates one group of objects from another. For statistical pattern recognition algorithms to be successful, there must be some criteria for them to base the allocation of different classes, and this is usually based on Cluster Analysis. A

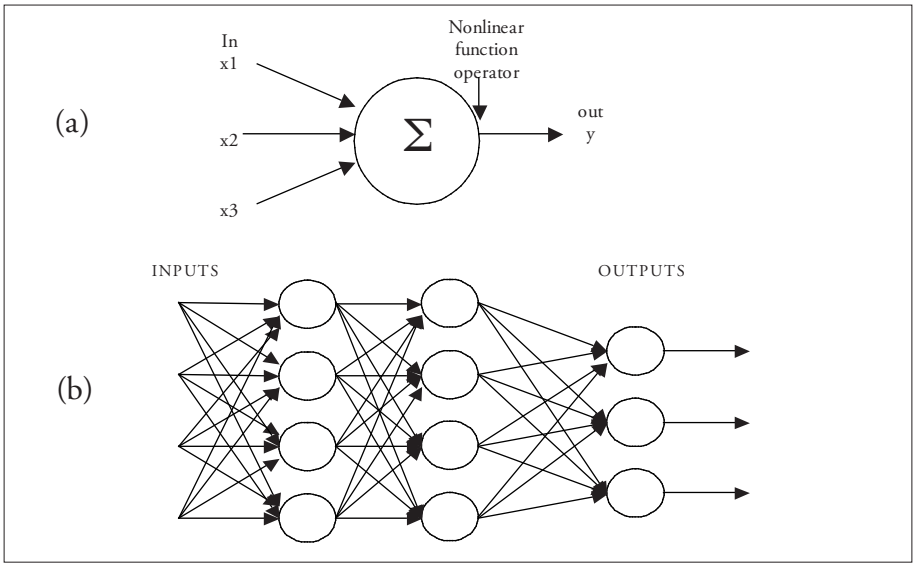

Fig. 6 *(a) Functional block of a node in a neural network, comprising a summation of several inputs followed by a nonlinear transform. (b) Architecture of a multiplayer perceptron, a commonly used neural network architecture*

given cluster of points representing a class has a center of gravity and a standard deviation distance that specifies the location and spread of the cluster. These values are obtained during the training phase of the algorithm, with replicate examples that are known to be members of a given class.

Artificial neural networks (ANNs), which have been used to analyze complex data and to recognize patterns in many other disciplines (Rumelhart, Hinton, and Williams, 1986), have shown promising results in recognition of volatile compounds and odors in electronic nose applications. When an ANN is combined with a sensor array, the number of detectable chemicals is generally greater than the number of unique sensor types. The advantages of ANNs over other methods of pattern recognition are numerous. Their performance is less affected by noisy or incomplete data. Another advantage is that they do not require linearly separable data sets.

Many ANN configurations and training algorithms have been used to build electronic noses including backpropagation-trained feed-forward networks; self-organizing maps (SOMs) (Kohonen, 1989); learning vector quantizers; fuzzy ARTmaps; Hamming networks; Boltzmann machines; and Hopfield networks. Classification systems applicable to electronic noses are reviewed by Horner (Horner, 1995). Modern neural networks may be either realized in hardware, or a simulation program running on a computer. The

software simulation, although generally slower, is cheaper and more flexible than the hardware counterpart. In both cases, the network consists of a series of processing nodes arranged into different layers, and as such, mimics the neurons in the brain. Each element is relatively simple, and is typically two function blocks as shown in figure 6(a).

The two most commonly used approaches for electronic noses are backpropagation-trained feed-forward networks and SOMs (Self Organizing Maps) (Ziegler et al., 1998). Backpropagation is a supervised algorithm that must be trained with labeled odours. It learns the relationship between the sensor values and the given odor labels. A SOM is an unsupervised algorithm that does not require predetermined odor classes for training. It essentially performs clustering of the data into similar groups based on the measured attributes or features that serve as inputs to the algorithm. It can be thought of as way of projecting multiple dimensions (often each dimension represents a different sensor output or a feature extracted from the sensor array) onto a two-dimensional output allowing the user to visualize the groupings and relationships of the odors or chemical volatile compounds.

4. APPLICATIONS

Electronic noses are in commercial use for a wide variety of odor and volatile compound applications. Their most popular applications currently are in food processing, environmental monitoring, medical diagnostics, process control, and fragrance development.

4.1 *Food Industry Applications*

Traditionally, food quality was assessed by panels of human experts and through application of analytical chemistry methodology. These are costly and time-consuming, and may be subjective. Electronic Noses do not replace sensory panels, but they have useful roles in the food processing industry, especially where simple comparisons need to be made. A host of applications tested include quality assessment in food production, inspection of food quality by odor, control of food cooking processes, inspection of fish, monitoring fermentation processes, checking for rancidity and spoilage, verifying sources of juice concentrates, fruit ripeness, and inspection of packaging material for malodors (Ali and O'Hare, 1997; Aparicio et al., 2000; Boerjesson et al.,

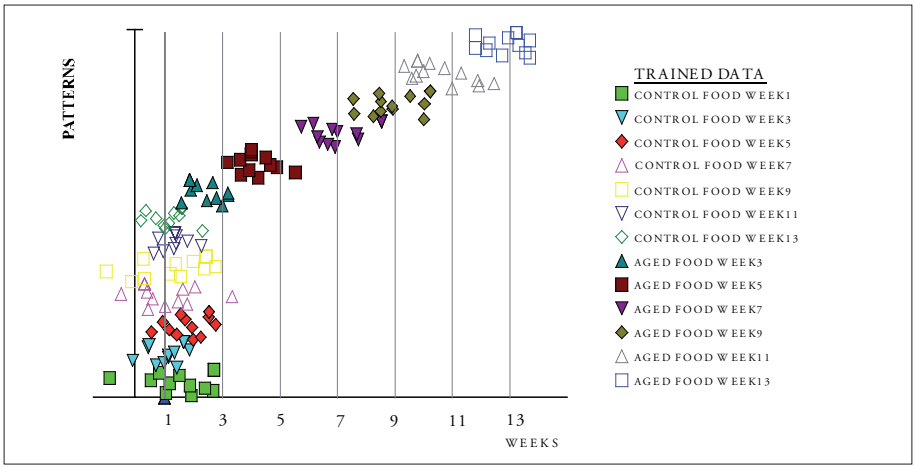

Fig. 7 *Use of a neural network to predict aging of biscuit samples. As the biscuits age, the fats oxidise and the measured odour pattern changes. The neural network is then used to map these changes so that the lifetime of the product can be predicted in terms of weeks of storage life. Each point corresponds to an odour measurement pattern, and these are mapped to time from the fresh control sample, by the neural network*

1996; Brezmes et al., 2000). Figure 7 illustrates an application using conducting polymer sensor arrays in investigating the shelf life of biscuits where oxidation of fats cause changes in smell and taste of the product prior to the onset of rancidity. In this case a neural network based prediction system was developed using radial basis function neural network architecture.

Typical applications of the technology in Food Analysis and Agriculture include

- Flavour and aroma
- Identification and classification
- Quality control of raw and manufactured products
- Freshness and maturity monitoring
- Microbial pathogen detection

There are requirements for on-line sensors, at-line sensors, as well as off-line sensors.

4.2 *Medical Diagnostic Applications*

Smell used to be a common diagnostic tool in medicine, and physicians were trained to use their sense of smell during their medical training. Latterly, odor

diagnostics have been relegated to secondary status as a diagnostic method. Electronic noses now offer the potential of a robust analytical approach to odor measurement for medical diagnostics (Gardner, Shin, and Hines, 2000; Gibson et al., 1997). Electronic nose technology has been used to examine odors emitted from the body such as from breath, wounds, and body fluids and identify possible problems, such as bacterial vaginosis (Chandiok et al., 1997) (Persaud et al., 2006). Breath analysis can be used to diagnose gastrointestinal problems, sinus problems, infections, diabetes, and liver problems. Infected wounds and tissues emit distinctive odors that can be detected by an electronic nose. Odors coming from body fluids can indicate metabolic problems as well as infections.

Other extremely applications are now being developed. These include monitoring mammalian cell growth (Bachinger et al., 2000), DNA detection (Clausen-Schaumann, Rief, and Seitz, 2000), and growth of bacterial cell cultures (Gardner et al., 1998).

4.3 *Environmental Applications*

Environmental applications of electronic noses increasingly include agricultural malodor applications. Malodors emanating from cow and pig slurries are an increasing source of environmental pollution as well as odor nuisance to human populations in the vicinity. Many substances produced during the anaerobic digestion of faeces have very low human olfactory thresholds and so are perceived as odor nuisances at very low concentrations in air. These include volatile fatty acids, p-cresol, amines, sulphides, disulphide, mercaptans and many heterocyclic compounds. It is now possible to correlate sensor responses to odor measurements derived from olfactometry using a human panel (Misselbrook, Hobbs, and Persaud, 1997; Persaud and others, 1996b; Persaud et al., 1996a).

Monitoring of indoor air quality is also an important application (Hathcock, Jr., 1999). A hybrid system was used aboard the Russian space station MIR to monitor air quality within the station (Persaud et al., 1999).

The problems of monitoring industrial chemical hazards may be addressed by array sensing technology (Huby, 1999; Khopkar, 1998; Stetter et al., 1984). Contaminating residues of insecticides (lindane and synthetic pyrethroids) and products from the leather manufacture (phenols, nitrobenzene, anilines) are often offloaded into streams or rivers, despite legislation and in disregard of the danger they present to the health of the population and the survival of fish and

flora. Electronic nose devices may prove promising in such monitoring applications (Baby, Cabezas, and Walsoe de Reca, 2000).

4.4 *Other Applications*

Many interesting applications are being developed and the state of the art is reviewed in Gardner and Persaud (Gardner and Persaud, 2000). They include fruit ripening, determining the origin of olive oil, spoilage of meat and fish, grain quality, fermentation monitoring, city pollution, fire detection (Scorsone et al. 2006), detection of potato pathogens (Stinson et al, 2006) and thousands of others. It is clear that the perception of the usefulness of such technology is high.

5. THE FUTURE

With a new technology, there are many unanticipated problems. It is a multidisciplinary area that attracts scientists, and companies from different areas of expertise. Perceived problems that need further development include sampling methodology, transferability of databases from one sensor array to another, repeatability and reproducibility over long term, the need for standardization of test protocols, and improved sensor technology. All of these areas are in active development, and it would appear that there is a long term future for this technology. At this point, many devices exist that are applicable to specific tasks. The "Electronic Nose" does not yet emulate a human nose, and the development of an "Artificial Nose", where the output code from the sensor array can be easily correlated to human sensory perception is still some time away.

ABSTRACT

There is worldwide interest in the potential of "electronic nose" technology for detection, measurement and discrimination of trace volatile chemicals – both odorous as well as non odorous chemicals. One approach involves the utilisation of cross-sensitivities between sensor elements in a chemical sensor array, combined with suitable information processing, resulting in enhancement of the discriminatory power of a small sensor array. The problems of objective odour measurements are immense, and this article attempts to put into perspective the design and performance of "electronic nose" instrumentation in analogy to biological chemoreception, with examples of how these instruments may be applied to real measurement problems.

RIASSUNTO

Le potenzialita' di un "naso elettronico" per la rivelazione, misura e discriminazione di sostanze chimiche volati in tracce – sia odorose che non – presenta ampio interesse nel mondo. Uno degli approcci prevede la misura di risposte incrociate da parte di elementi sensibili in una batteria di sensori chimici, integrata da un sistema di elaborazione delle informazioni ed avente come risultato un miglioramento del potere discriminatorio da parte di una piccola serie di sensori. I problemi della misura strumentale degli odori sono notevoli e questa presentazione e' un tentativo di confronto fra il progetto e le prestazioni di un "naso elettronico" ed il sistema biologico di chemorecezione, fornendo allo stesso tempo esempi di applicazione di tali strumenti a concreti problemi di misura.

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Conclusions

The reports here collected have presented the main open questions in olfactory research and provide information on the state of the art in this field. The lecture of Linda Buck has provided a compendium of the biochemical processes occurring in the olfactory system from the initial recognition of chemical stimuli by olfactory receptor proteins to the very complex mechanisms of signal processing in the olfactory cortex, thus sheding light for the first time on how odour images are formed in the brain. The second contribution by Paolo Pelosi was focused on soluble components of the olfactory system, odorant-binding proteins, whose physiological function still remains one of the major unsolved question in olfaction. The lecture of Aldo Fasolo has illustrated the plasticity of the olfactory system and the huge potentialities and implications related to its unique developmental mechanisms. Walter Leal has shown how research in olfaction can provide information that can be applied to insect population control in agriculture, providing economical benefits, while protecting the environment. The closing lecture of Krishna Persaud has presented the state of the art on artificial noses and the possibility of an instrumental measurements of odours, with interesting applications in the fields of food quality, environmental monitoring and most recently in clinical analysis.

The field of olfactory research, that got its major impulse from the discovery of olfactory receptors by Linda Buck and Richard Axel in 1991, proves after 15 years to be still very active and full of exciting perspectives.

Conclusioni

Le relazioni qui raccolte hanno presentato i principali problemi nella ricerca sull'olfatto ed hanno fornito informazioni sullo stato dell'arte in questo campo. La conferenza di Linda Buck ha fornito una sintesi dei processi biochimici che avvengono nel sistema olfattivo dal riconoscimento iniziale degli stimoli chimici da parte dei recettori olfattivi ai complessi meccanismi di eleborazione dei segnali che avvengono nella corteccia olfattiva, svelando per la prima volta come le immagini olfattive si formano nel cervello. Il secondo contributo di Paolo Pelosi ha avuto come oggetto componenti solubili del sistema olfattivo, le proteine leganti gli odori, la cui funzione fisiologica rimane una delle principali questioni aperte nello studio dell'olfatto. Aldo Fasolo ha quindi illustrato la plasticita' del sistema olfattivo e le enormi potenzialita' ed implicazioni derivanti dai suoi peculiari meccanismi di sviluppo. Walter Leal ha poi mostrato come le ricerche nel campo dell'olfatto possano fornire informazioni utili per il controllo di popolazioni di insetti in agricoltura, con conseguenti benefici economici ed un maggiore rispetto dell'ambiente. La presentazione finale di Krishna Persaud ha fornito lo stato dell'arte sui nasi artificiali e sulle possibilita' di una misura strumentale degli odori mediante interessanti applicazioni nel controllo di qualita' degli alimenti, del monitoraggio ambientale e piu' recentemente di analisi cliniche

Il campo della ricerca olfattiva, che ricevette il maggior impulso dalla scoperta dei recettori olfattivi da parte di Linda Buck e Richard Axel nel 1991, rimane dopo 15 anni ancora molto attivo e fonte di interessanti prospettive.