ENHANCEMENT, MODULATION AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF MURINE OLFACTORY NEURONS TO ODORANT STIMULATION *IN VITRO*

Except where reference is made to the work of others, the work described in this dissertation is my own or was done in collaboration with my advisory committee. This dissertation does not include proprietary or classified information.

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ENHANCEMENT, MODULATION AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF MURINE OLFACTORY NEURONS TO ODORANT STIMULATION *IN VITRO*

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VITA

Nilmini Viswaprakash (nee Subramaniam), daughter of Ambalavanar Rajalingham Subramaniam and Nagapooshany Subramaniam (nee Sinnathamby), was born in Matale, Sri Lanka. She earned her bachelor's Degree in Zoology from University of Madras, India and subsequently her Masters degree in Marine Biology and Oceanography from the Centre of Advanced Studies in Marine Biology and Oceanography, Annamalai University, India. Nilmini was appointed by the ministry of education, Srilanka as Fisheries Science and Human biology teacher in the Republic of Maldives, where she held the positions of head of the department, Secretary of teachers association, Panel member of fisheries curriculum. In 1996, the family migrated to New Zealand where she acquired a diploma degree in business computing and was employed as a customer support technician for Hewlett Packard computers, Auckland, New Zealand. Nilmini joined the Doctoral program in Biomedical Sciences, Auburn University to pursue research studies in electrophysiology under the guidance of Dr. Vitaly Vodyanoy in fall 2001. Nilmini was born with 6 other siblings and has five sisters and one brother. She is married to Nanduri Viswaprakash and has two sons Ajitan and Ajay.

Dedication: With all my love to my precious parents in the name lord Krishna, whom I believe is The Creator, The Maintainer and The Destroyer of this universe.

DISSERTATION ABSTRACT

ENHANCEMENT, MODULATION AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF MURINE OLFACTORY NEURONS TO ODORANT STIMULATION *IN VITRO*

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The objectives of this study is to electrophysiologically characterize cultured olfactory epithelium (OE) and to compare with that of rapidly isolated OE; to establish the $G_{\alpha i}$ involvement in the modulation of adenylyl cyclase (AC) and to demonstrate the participation of zinc in odorant stimulated enhancement of smell. The murine family has a well developed sense of smell and is widely used in the field of olfactory research. Producing a long lived, readily accessible, economically feasible and physiologically viable cultured OE model would be ideal, to meet with specific requirements and nature of the research.

The cultured OE of different ages were exposed to charcoal-filtered air, individual odorants, and an odorant mixture of (+)carvone, (-)carvone, eugenol, and ethyl butyrate at

varying concentrations or with varying inter pulse periods to explore the system's ability to respond to and adapt to odorant presentation.

The study also involved with $G_{\alpha i}$-Protein Inhibition of olfactory signal transduction. The whole-cell voltage clamp recordings of odorant-stimulated olfactory neurons indicate that endogenous G_i protein negatively regulates odorant-evoked intracellular signaling. Rat OE were subjected to whole cell recordings with holding membrane potential of +/- 57 mV. The patch electrode (resistance of 8±3MΩ) was filled with intracellular fluid (ICF) in the presence or absence of antibodies directed against the $G_{\alpha s/olf}$-subunits (Santa Cruz Biotech.), antibodies raised against $G_{\alpha i}$-subunits (Calbiochem) or control antibodies.

In addition to G proteins involvement in the enhancement, modulation and inhibition of Odorant signals, there is evidence that metals like zinc enhances odorant induced response of Rat OE are exposed to crystalline metallic nanoparticles which are found in proteon nucleating centers (PNC) and metallic Zinc nanoparticles (artificial PNC). EOG recordings were made from dissected rat olfactory receptor neurons under control conditions (in the presence of extra cellular fluid -ECF) and experimental conditions (in the presence of PNC and Zn and Cu).The metal nano particles were integrated with the odorants of interest and were delivered onto the odor receptors.

Our results show that in cultured tissues the characteristics of the EOG responses are similar to that of standard EOG responses of dissected OE. Cultures one week old or less (n = 8) showed rapid rise times but prolonged decay times. Cultures aged 13 to 15 days in vitro (n = 8) showed both rapid rise and decay times. Although all cultures responded to the four individual odorants in equimolar concentrations, their response to

each odorant was variable; ethyl butyrate elicited the strongest response on average. High frequency stimulation with inter pulse intervals of 200 ms, the EOG responses appeared to be a summative effect due to closely spaced inter stimuli intervals. At longer durations of the inter-stimuli intervals (800 ms), adaptation was clearly apparent with the second and succeeding responses of lower amplitude than the initial response. This trend continued until the duration of the inter-stimuli intervals reached 20 seconds, where the second and succeeding response amplitudes were equal to that of the first EOG amplitude. These results extend the characterization of our olfactory epithelium-olfactory bulb organotypic culture system and suggest that olfactory epithelium passes through an immature stage in the week following explanation and culturing.

The rapidly isolated rat OE with intracellular microinjections $G_{\alpha s/olf}$ antibodies inhibited current responses elicited by odorants. In contrast, antibodies raised against $G_{\alpha i}$-subunits caused a strong enhancement of the odorant-induced currents. When IgG or anti-olfactory marker protein antibody was substituted for G-protein antibodies, the odorant-induced currents were not significantly changed. Odorant-induced responses were dose-dependent in the range of 0-16 mM.

The metallic nanoparticles enhanced the EOG potentials up to 10 fold and single cell current responses elicited by a mixture of odorants up to 6 fold. Also incorporation of the metal nanoparticles with the odorants and delivery of the odorant /metal nanoparticle mixture to the odor receptors showed significant stimulation of the olfactory neurons.

Our results show a promising future in yet another area in the field of olfaction where crystalline metals can be used to enhance and sustained olfactory responses.

Style manual used: Chemical Senses

Computer software used: Microsoft office; Microcal Origin 6.0; Adobe Photoshop

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I wish to thank my beloved mother and father who are my inspiration in life. I miss my father on my triumphant occasion. The sacrifices they underwent to provide a sound education, encouragement and belief in me has made me a successful person. I also wish to thank my sisters and brother who have constantly been there for me.

Words cannot express my sincere gratitude to my mentor Dr. Vitaly Vodyanoy. I always felt it as a privilege to have come to know of him and to be taught and trained by him. I couldn't thank him enough.

I received utmost support from my collaborators and committee members, Dr.E. Morrison, Dr. E. Josephson, Dr. Anthony Moss and Dr. John Dennis. I thank them whole heartedly for their guidance and patience throughout the course of my project. Special mention goes out to Dr. Josephson for providing me with material for this study. Thanks also go out to Solomon Yilma for training and assisting me with patch clamp technique.

I owe my deepest gratitude to my husband Prakash and my sons Ajitan and Ajay for their patience, understanding and encouragement throughout my research years. I thank all my wonderful friends in Auburn with a special note to Anjani Kumar for initiating me with the process of self realization and Rajesh for being there for me when I needed the most.

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1. INTRODUCTION

In the human world, olfaction serves to heighten or discriminate our aesthetic sense, while in other animals it is an important survival sense, upon which the well-being and sometimes the safety of the animal itself is dependent on. The partial or complete loss of smell (anosmic) affects approximately 2.5 million people in U.S.A and poses serious health risks as the presence of rancid food; toxic odors and smoke often go undetected. The clinical treatment of anosmic will require a better understanding of the mechanisms that regulate differentiation, proliferation and the injury response of the precursor, the multi-potent neuronal stem cells in the olfactory epithelium (OE). Olfactory receptor neurons (ORN) are the only mammalian neurons which undergo continuous neurogenesis throughout their life, suggesting that a neuronal stem cell exists in this system. In vitro systems of ORNs would greatly facilitate studies of both neurogenesis and physiological/biochemical activities of the olfactory sensory cells.

The olfactory system sits at the interface of the environment and the nervous system and is responsible for correctly coding sensory information from thousands of odorous stimuli (Figure1). Many theories existed regarding the signal transduction mechanism that mediates this difficult task.

1.1 Olfactory Epithelium

ORNs are bipolar, with dendrites running towards the surface of the OE and axons towards the OB (Graziadei et al 1979). The dendrites consist of cilia, lining the

olfactory passage and dangles in the mucus secreted by mucus secreted by the supporting cells. The initial events of olfactory transduction take place in the cilia. (Figure 2) Electrophysiological studies indicate olfactory transduction; the initial phase of odorant perception takes in the cilia.

1.2 Signal transduction

Olfactory signal transduction is initiated when odorants bind to the G Protein coupled receptors located in the cilia following which a cascade of events are triggered resulting in the stimulation of AC, formation of cAMP, influx of cations and efflux of Anions leading to the generation of a graded receptor potential and depolarization . Electrophysiological and biochemical studies indicate that cAMP is the key messenger during olfactory transduction (Figure 3)

Volatile chemicals bind to Olfactory receptors, stimulating G_{olf} activation of AC, thereby increasing intracellular cAMP levels, which triggers the opening of cyclic-nucleotide gated cation channels(Nakamura 1987) .An inward transduction current caused by Na+ and Ca++ (Frings et al., 1995; Gavazzo et al., 2000), along with Ca+ induced Cl_ current depolarize the cilia Kleene and Gesteland, 1991b; Kurahashi and Yau, 1993).This membrane depolarization (Odorant induced current) can be measured by Whole cell recordings in isolated olfactory epithelia. The depolarization spreads to the dendrite and the cell body of the neuron, electrotonically may trigger Action potential.

1.3 G Protein

G proteins are seven-Transmembrane spanning domain receptors. G Protein mediated secondary transduction system serves multiple functions in the Eukaryotic

organisms. Almost 80% of the pharmaceutical drugs acts through G Protein receptors and will remain as the prime target in drug manufacturing. Biochemical studies indicate the involvement of G proteins in olfactory transduction. The excitatory $G\alpha_{olf}$ which is expressed only in ORNs stimulates AC where as the inhibitory Gai generally modulate the production cAMP by acting AC (Sinnarajah et al 1998).

G- Proteins are classified into 4 families based on their structural and functional resemblance of the α subunit.: Gs, which has a stimulatory effect on adenylyl cyclase (AC); Gi/o, which has an inhibitory effect on AC and mediates potassium channel activation and voltage dependent Ca2+ channel inhibition; Gq/11, upon coupling to receptors stimulates β isoforms of phospholipase C (PLC); and G12/13, which mediates small GTPase Rho. (Wettschureck 2004)

The G-Protein signaling mechanism is the predominantly functional second messenger system existing in all the eukaryotic organisms. Mammalian olfactory sensory cells express many types of heterotrimeric G- Proteins, G- Protein coupled receptors (GPCR) and effectors. GPCRs are the largest cell surface receptors and an array of hormonal, neurotransmitter functions and sensory perceptions such as taste, Olfaction and light involve GPCR.

GPCRs trigger G-Proteins, upon which the α subunit detaches from β and γ , binds and hydrolyses guanosine triphosphate (GTP), [Hepler & Gilman, 1992, Neer, 1995, Gudermann et al., 1996 and].

1.4 cAMP

cAMP based transduction is best triggered by fruity, floral, and herbaceous odors.

Electrophysiological studies provided evidence for the role of cAMP in odorant detection. cAMP the neurotransmitter, directly guards the cyclic nucleotide channels allowing the entry of cations which eventually causes depolarization.

1.5 Adenylyl cyclase

A membrane bound enzyme that catalyzes the formation of cAMP. Ac is one of the most Important second messengers which involves in the regulation of multiple cell functions Including growth ,Proliferation, differentiation , secretion and modulates many cellular processes in response to extra cellular signals such as hormones and neuro transmitters

The subunits of G Protein stimulate the enzyme Adenylyl cyclase which catalyzes the conversion of +ATP into cAMP during transduction. There are around nine known isoforms of AC expressed in different tissues. G_{olf} and AC3 are co localized in olfactory cilia, indicating that G_{olf} may mediate the activation of AC3.

1.6 Olfactory Phosphodiesterase

During transduction formation and degradation of cAMP constantly takes place. PDEs activities are regulated by calcium, cyclic nucleotides, and phosphorylation and this may have an odorant involved indirect negative feed back on cAMP indicating a possible site of regulation for the odorant-induced cAMP response.

1.7 Channels

Ligand gated ion channels are expressed in olfactory cilia which allows movement ions which leads to the generation of graded potential and signal amplification. cAMP gated CNG channels allows the influx of cations which leads to

receptor potential and Ca^{2+}-gated $Cl^{=}$ channels promote the efflux of Cl- that amplifies the receptor current . The expulsion of Ca^{2+} ions takes place through Na^{+}/Ca^{2+} exchange mechanism during termination of transduction.

1.8 Other signaling cascades in olfaction

There is evidence for the existence to other signaling pathways active in odorant transduction.

1.8a Inositol-1, 4, 5-trisphosphate (IP3)

In some vertebrate species, odorants have been shown to elicit rapid increases in IP3, as well as in cAMP, implicating that two separate signal transduction pathways exist in olfactory neurons (Breer and Boekhoff, 1992; Schild and Restrepo, 1998). The odorants citralva and eugenol appear to increase cAMP, whereas some other odorants such as lyral, lilial, and ethyl vanillin have been shown to elicit IP3 increases in biochemical assays (Breer and Boekhoff, 1992; Schild and Restrepo, 1998). Binding of chemical stimuli to olfactory G protein-coupled receptors (GCRs) has generally been considered to lead to accumulation of cAMP or IP_3 in olfactory neurons and activation of cAMP- or IP_3-gated channels, causing cell depolarization and olfactory nerve responses. In addition, a response of an olfactory neuron to an odorant contains a large component via neither olfactory GCR nor second messenger-dependent pathways. We discuss here various pathways in the olfactory transduction.

1.8b cGMP

Cyclic GMP is the predominant second messenger in visual signal transduction. Studies show that cGMP may also play an important role in odorant transduction process.

A slower and sustained formation of cGMP was observed in olfactory tissues. This may be due the fact that cGMP is involved with long-term cellular events such as desensitization. cGMP levels are regulated guanylyl cyclase which is in turn is activated by gaseous messengers such as NO or CO.

1.9 Calcium

Calcium acts as a two faced messenger in olfactory transduction by initiating the transduction process and by a negative feed back in the down regulation and termination of signal transduction.

1.10 Desensitization

Down regulation of olfactory signal transduction is mediated through receptor phosphorylation, (Boekhoff et al 1992) deactivation of G Protein (Simon et al 1991) modulation of AC activity (Sklar et al 1986), and activation of Phosphodiesterace. Cyclic GMP may also play a role in down regulation. Zufall et al (1998) showed that cGMP mediated a long-lasting form of odor adaptation in tiger salamander. In addition modulation and adaptation of CNG channels also contributes towards desensitization.

The ability of olfactory neurons to undergo neurogenesis lead to the development of an array of culture models to study their properties in vitro. The stability of the OE and OB co culture developed in this study is the first culture model with olfactory synapse between olfactory receptor neurons and olfactory bulb. Such a preparation would provide a rich, yet accessible, system to be used in the field of olfactory, pharmacology and biosensor research.

In vitro models, such as cell and organ culture, have advantages over their counterparts which make them preferable for experiments with specific requirements.

Physiological studies that require the anesthetization of intact animal may alter cellular responses. Bleeding and shock during dissection can interfere with optical or electrical recordings. Intact cellular preparation may hinder anatomical studies due to tissue density which interferes with the transillumination required to visualize histological structures. In vitro culture models allows easy access to the system for making physiologic recordings, visualizing changes in functions and morphological features , application of drugs and other agents, (Suzuki,2000)

ORNs exhibit a better survival and maturity in the presence of the olfactory bulb {Chuah, 1983; Verhaagen, 1990}. If a culture model survives for months instead of days or weeks and physiologically active, it is possible to subject them for studies which require large number of OE of the same stem cell origin, comparative studies and the long-term effects of treatments on olfactory structures.

Limitations of the traditional way of using rapidly isolated OE and conventional methods of short lived OE culture with in obtaining continuous and large quantity of olfactory tissues. With improved culture techniques, it is possible to produce large quantities of long lived cultured OE for future applications in the field of olfactory research. Successful mouse models of transgenic expression, knockout species, and altered components of G-Protein system have been produced in the past to answer question in olfaction. These mouse models have significant contribution in identifying the physiological role of G- Protein signaling system and thereby in the identification of drug targets.

We also investigated the possibility of obtaining electrophysiological recordings from cultured mouse OE in the whole-cell configuration, a technique that allows the measurements of electrical activity from a single olfactory neuron. Using electrophysiological techniques to study the similarities in the functional properties of OE and for a better understanding of the molecular mechanisms of olfactory transduction, it is therefore of great importance to be able to obtain electrophysiological recordings from cultured mouse OE (Figure 4, figure 5).

The past two decades proved to advances in our understanding of olfactory transduction with the realization that G proteins and the coupling receptors are involved in odorant detection, cAMP cascade is the predominant mechanism of transduction and the excitatory effect $G\alpha_{olf}$ in the production of cAMP. However it remains to be determined the involvement of other transduction cascades, the G protein–coupled receptors and the inhibitory effect of Gαi on cAMP production which leads to modulation of olfactory responses. With the realization of the presence of Gαi in the OE and the inhibitory action it has on Ac and thereby on cAMP production it is expected that Gαi will negatively act on AC upon binding to odorants and thereby inhibiting or modulating the formation of cAMP Biochemical studies indicated Gαi mediated inhibition in rat olfactory ciliary preparation. In this study rat OE are subjected to electrophysiological studies to establish that Gαi has a direct involvement in cAMP reduction by acting on AC during olfactory transduction.

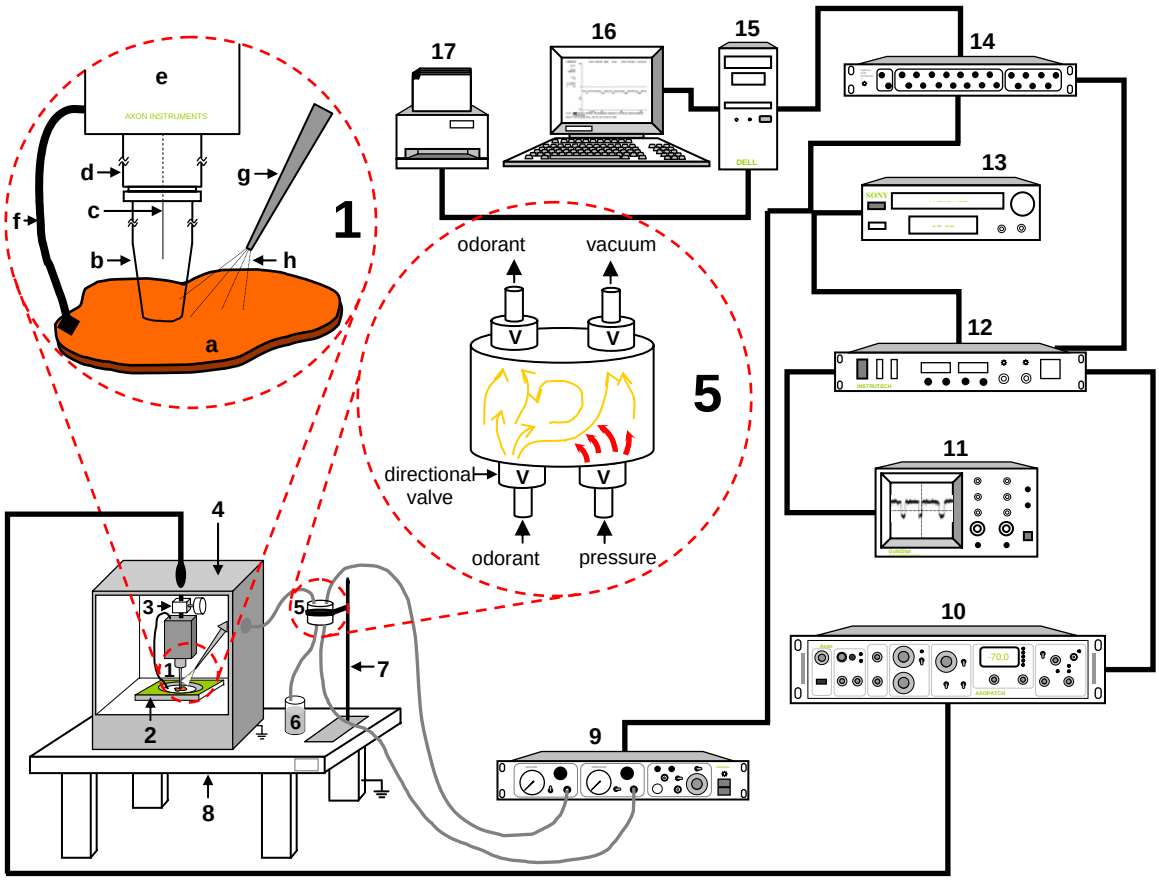
However a large portion of the G-Protein function is yet to be studies or revealed. One such function is the involvement of zinc with G protein during transduction which results in enhancement of odorant responses.

Studies shows odorant evoked olfactory responses in rat olfactory receptor neurons by employing crystalline metallic nanoparticles which are found in proteon nucleating centers (PNC) derived from a number of biological sources and metallic Zinc nanoparticles (artificial PNC) .Olfactory stimulation of Rat OE was measured using EOG (6) and whole cell recordings (7). The physiological effects of using certain metals in enhancing the olfactory responses were studied. Induction of PNC and Zn enhanced odorant evoked olfactory responses.

The metallic nanoparticle enhanced the EOG potentials and single cell current responses elicited by a mixture of odorants. The method for incorporation of the metal nanoparticles with the odorants and delivery of the odorant /metal nanoparticle mixture to the odor receptors also found result in significant stimulation of the olfactory neurons. Odorant-induced currents suggest that metallic nanoparticles are physiologically significant for olfactory signaling. The practical applications of this invention of enhancing the response of olfactory receptor neurons to odorants are significant. Odorant-induced currents suggest that metallic nanoparticles are physiologically significant for olfactory signaling. The behavior we observed in rat olfactory tissue is valuable because it reveals one of the important properties of the olfactory system. The study will help us to better understand the dynamics of olfactory enhancement and employ it in constructing a practical olfactory response model. Aside from providing indirect evidence for the involvement of a metal ion in the transduction process, metal

binding could also be useful in fragrance design if it were Possible to build into the molecule to increase odorant potency.

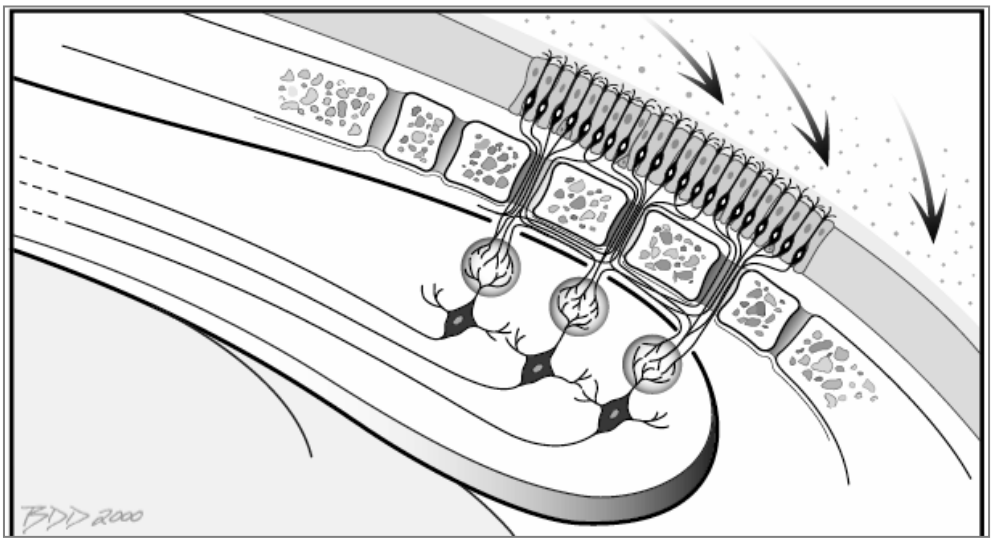
A. Experimental Setup



1. Patch-clamp setup: a. Olfactory tissue; b. Glass micro-electrode; c. Silver/silver-chloride wire; d. Glass micro-electrode holder; e. Head stage; f. Ground wire; g. Odor applicator / puffer; h. Odor stream

2. Perfusion chamber; 3. Micro-manipulator; 4. Faraday-box; 5. Volatile odor-air mixing chamber; 6. Liquid odorant container; 7.Metal stand; 8. Isolation table; 9. Pneumatic pico-pump; 10. Patch-clamp amplifier; 11. Oscilloscope; 12. Analog-digital converter; 13. Video Cassette Recorder (VCR); 14. Digidata 1200A digital interface box; 15. Computer CPU; 16. Computer monitor and keyboard; 17. Laser printer.

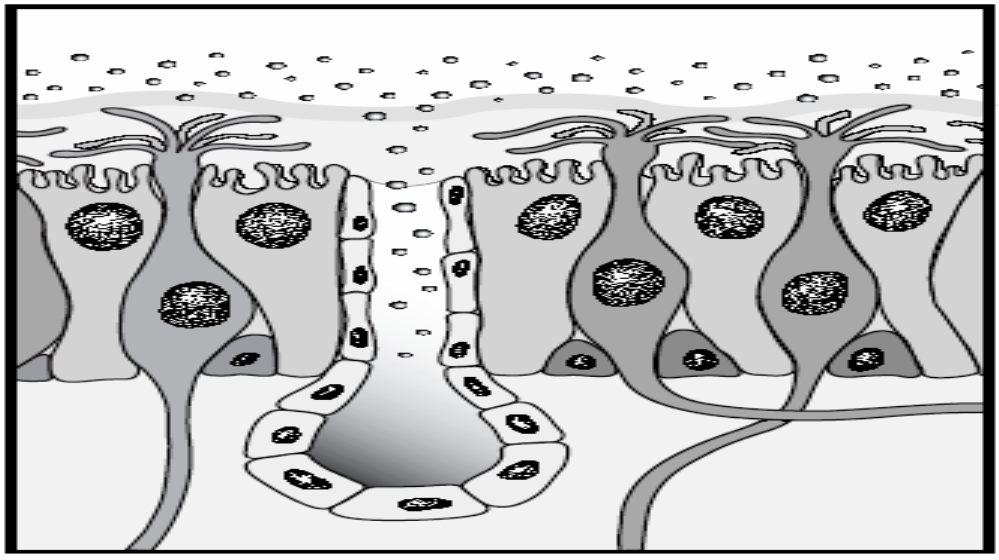
Olfactory system
Figure 1



Courtesy of: Dr. Vitaly J Vodyanoy

OE cellular arrangements

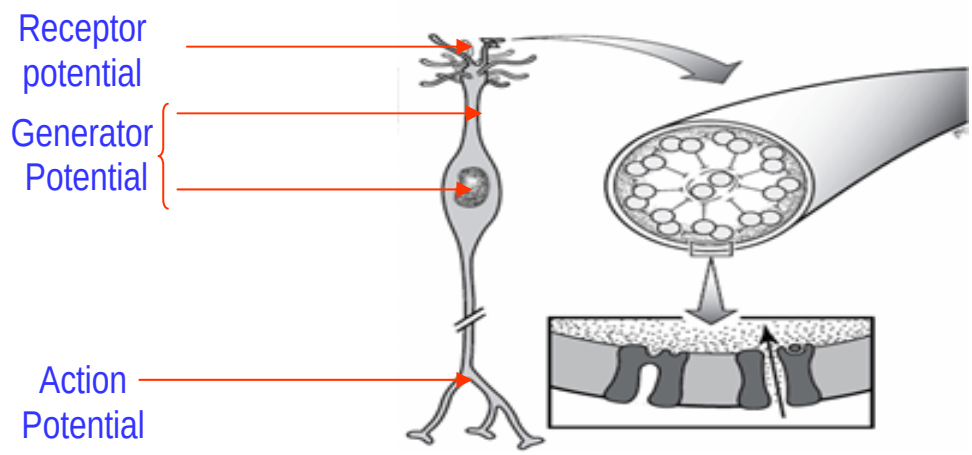
Figure 2



Courtesy of: Dr. Vitaly J Vodyanoy

Transduction and signaling regions

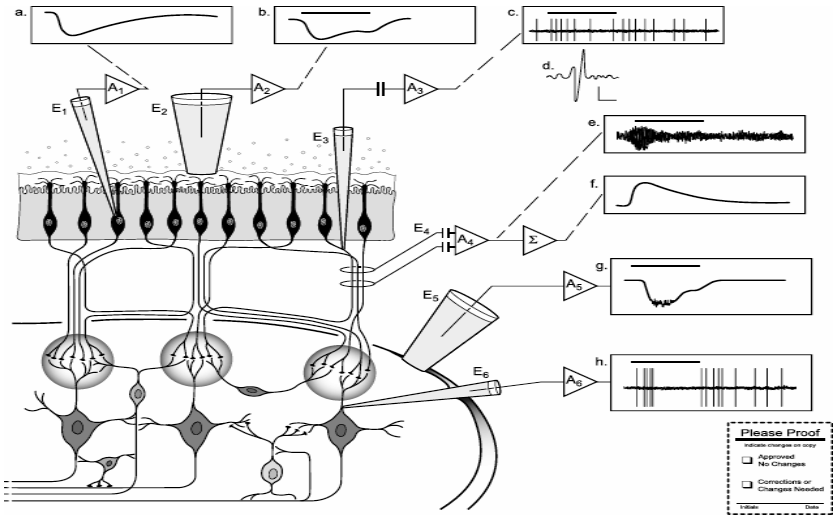
Figure 3



Courtesy of: Dr. Vitaly J Vodyanoy

Measuring electrical activities using electrophysiological techniques

Figure 4



Courtesy of: Dr. Vitaly J Vodyanoy

Figure 5

Electroolfactogram recording

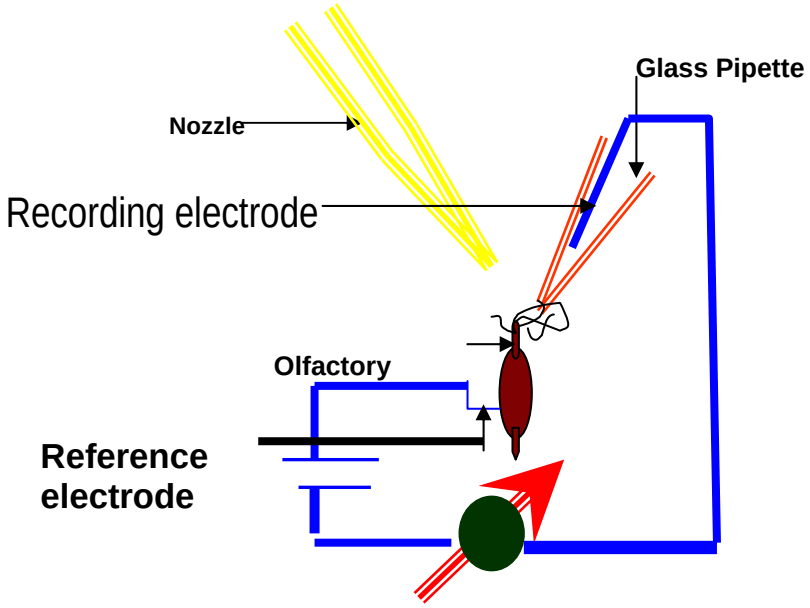
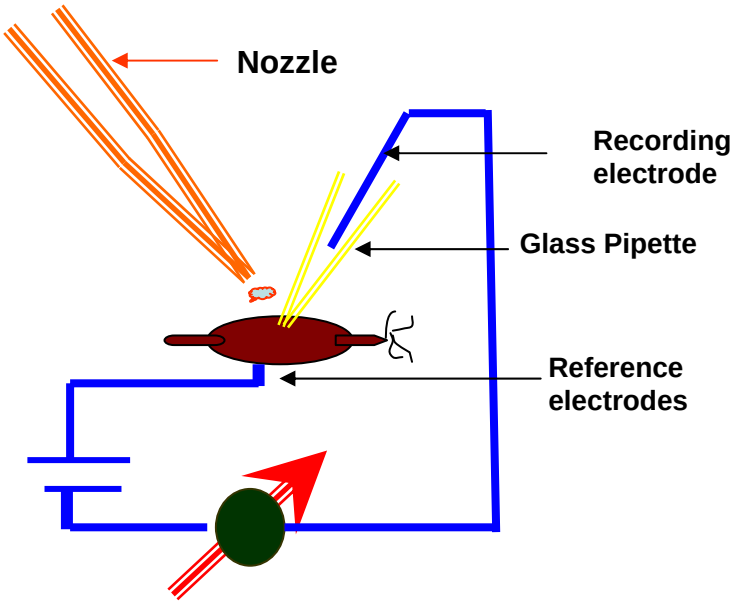


Figure 6

Whole cell recording



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2. LITERATURE REVIEW

2.1 Olfaction

The sense of smell is part of our chemosensory system. Olfactory sensory neurons help us in the perception of smell and taste flavors. Once the receptor cells detect volatile odorant molecules they send messages to the brain, where odor perception take place. The olfactory system exhibits many complex mechanisms to accomplish the difficult tasks of identifying various odors with different configurations, concentrations.

In mammals, odorant inhalation takes place through the nose. Order molecules pass into the olfactory nares upon which they contact the olfactory epithelium. Olfactory receptor neurons (ORNs) transduce chemical signals from the odorants into electrical signals which then travel along the axon to the olfactory bulb where neurons converge to form glomeruli. In the glomulerus, the axons meet the dendrites of mitral cells. Mitral cells send their axons to the cortex of the brain. Malfunction of the olfactory system, which can occur as a result of traumatic brain injury, inhalation of toxic fumes, or neurological diseases such as Parkinson's disease, Alzheimer's disease and can cause anosmia.

2.2 Olfactory Research

Olfaction has been a fascinating research topic for many years. Since Ottoson (1959) discovered the slow potential across the olfactory epithelium (OE),

electroolfactogram (EOG). When a puff of odor enters the olfactory region, the surface generally goes negative from its normal potential and then recovers. Many other relative phenomena have been described by Gestland R.C et al (1965), Higashino, S, S (1956), Shibuya, T et al (1963) and Takagi et al (1960).

The study of olfactory transduction has been difficult because of the delicate and minute morphology of ORNs. The pioneer work by Cole (1936), lead to the development and design of patch clamp techniques. This revolutionized electrophysiological techniques for the study of olfactory transduction (Anholt, 1991; Firestein, 1991). A combination of molecular, electrophysiological, and cell biological approaches was required to delineate odorant transduction.

2.3 Olfactory Transduction

The initial events of odor perception are controlled predominantly G protein cascades. A number of other signal cascades are also activated during transduction. These secondary cascade systems are only beginning to be understood, and many controversial issues remain to date (Gold, 1999). Odorant signal transduction is initiated when odorants binds to specific receptors on the cilia of ORNs (Buck, 1996; Rhein, 1980) and act through G-protein receptors to stimulate adenylyl cyclase (AC) (Pace et al; 1985).

G proteins transduce messages from receptors into cellular responses mediated by effector enzymes and ion channels. G-protein coupled receptors are a pharmacologically distinct protein family with at least 450 genes identified to date. Olfactory G-protein coupled receptors trigger the biochemical synthesis of cAMP and IP_3, triggering the

opening of cation channels that ultimately lead to action potentials and signaling. (Anholt, 1993).

Electrophysiological and biochemical studies show that cAMP is the key messenger in olfactory transduction (Pace et al 1985, Jaworsky 1995). During olfactory transduction the concentration of cAMP in the cilia increases, there by resulting in an influx of sodium and calcium ions via cyclic nucleotide-gated channels (Firestein, 1989; Nakamura, 1987). The immediate biophysical response is the generation of a graded receptor potential (Getchell, 1978; Ottoson, 1945).

2.4 Other Cascade Systems

Other cascades are activated during odorant detection. These include pathways activated downstream of the cyclic nucleotide-gated channel through calcium permeability (Kaupp, 1991). Odorants also increase the production of inositol-1, 4, 5-trisphosphate (IP_3) (Miyamoto, 1990), and cGMP production (Ingi, 1996; Verma, 1993). Interestingly, the ORN's response to odorant-induced cGMP production is much slower than the cAMP or IP_3 responses. Thus, the cGMP response does not appear to function in the immediate detection phase of olfaction but rather in adaptation or the modulation of the cellular response during longer exposures to odorants (Moon, 1999; Breer, 1992). Olfactory receptors (ORs) are the largest gene family in the mammalian genome. In 1991, Linda Buck and Richard Axel discovered the family of transmembrane order receptor proteins and some of the genes that encode them. The discovery was a major breakthrough in the understanding of the olfactory system (Buck 1991). The proteins

contained the 7 helical transmembrane structure and sequence similarity to other members of the "G-protein" linked receptor family.

2.5 Related Studies

The first direct biochemical studies to report an odorant-induced cAMP response in olfactory system was from isolated cilia of both frog and rat (Pace, 1985; Sklar, 1986). The odorant-stimulated cAMP production occurred only in the presence of GTP, suggesting the involvement of receptors coupled to G proteins. Electrophysiological studies provided further evidence for the key role of cAMP in odorant perception. Electrical recordings from excised cilia demonstrated a cAMP-gated conductance. It is proposed that odorant stimulation would increase intracellular cyclic nucleotide concentration to gate a cationic conductance, initiating a depolarizing response .Kinetic studies of odorant-evoked currents recorded in the whole-cell configuration (Firestein, 1990) suggested that the latency of the odorant response supported a role for a second messenger such as cAMP. Further biochemical characterization using isolated rat olfactory sensory cilia showed that cAMP was mainly produced by fruity, floral, and herbaceous odors (Sklar, 1986).

The time scale for the production of cAMP was measured by the kinetics of odorant-induced changes (Breer, 1990; Boekhoff, 1990). Cilia and odorant solutions were reacted together using computer-controlled mixing, cAMP was produced rapidly and transiently in response to odorants, with increases evident as early as 25 ms. Fruity odors were able to stimulate cAMP production at concentrations as low as 10 nM, whereas others, such as putrid odors, had no effect, even at higher concentrations. There are at

least nine identified isoforms of adenylyl cyclases (ACIII), which generate cAMP (Hanoune, 2001). Bakalyar & Reed (1990) cloned ACIII. Northern blot analysis revealed that ACIII mRNA was enriched in the olfactory epithelium and that ACIII message disappeared after bulbectomy. The low basal activity of ACIII may be relevant to its role in sensory transduction. G_{olf} and ACIII have been localized to olfactory cilia, indicating that G_{olf} may mediate the activation of ACIII (Menco, 1992).

To evaluate the role of ACIII in the olfactory transduction ACIII–null mice were stimulated with odorants which completely eliminated EOG. Moreover, odor-dependent learning was impaired in these mice. Interestingly, both cAMP dependent fruity odors and IP3 dependent putrid odors failed to evoke any response in these animals. A pharmacological study showed that adenylyl cyclase antagonists reversibly inhibit EOG even to putrid odors thought to be transduced by IP3 (Chen, 2000). These results confirmed earlier biochemical studies that cAMP is essential for the initial phases of odorant transduction. IP3 was therefore appearing to play more of a modulatory role in the odorant transduction in mammals. There are about 7 different gene families of PDEs whose activities are regulated by calcium, cyclic nucleotides, and phosphorylation (Beavo, 1994; Beltman, 1993). Several forms of cAMP-PDE are expressed in rat olfactory cilia (Borisy et al, 1991 & 1993). A calcium/calmodulin PDE (CaM-PDE) is exclusively found in ORN which has a high affinity for cAMP and could be activated by odorants in response to ciliary calcium increases. Cloning of the high-affinity PDE revealed it to have a higher affinity for (Yan, 1995).

Olfactory neurons also express cGMP-stimulated phosphodiesterase (PDE_2) (Juilfs, 1997). In these specific ORNs, guanylyl cyclase type-D (GC-D) is also expressed, suggesting that it may play an important role in odorant transduction for a specific subset of responses. PDE2 and GC-D are both expressed in olfactory cilia of these neurons; taken together, these data strongly suggest that selective compartmentalization of different PDEs and cyclases is an important feature for the regulation of signal transduction in ORNs. In ORNs; ion channels are expressed in cilia, where they generate and amplify the odor-induced receptor current. Ca^{2+} signals generated by CNG channels play an important role in amplification of sensory transduction in vision and olfaction. The ability of CNCs to conduct Ca^{2+} determines both the rise time and the amplitude of the odorant receptor current, as well as its termination after the stimulus. Ca^{2+}-gated $Cl^{=}$ channels are triggered by Ca^{2+} influx through CNCs and cause a depolarizing $Cl^{=}$ efflux that amplifies the receptor current (Lowe, 1993).

Excitation of an olfactory neuron generates a receptor potential, and when the membrane potential reaches the firing threshold, Na^{+} channels activate and initiate spike generation. The Na^{+} currents are increased via cGMP-dependent phosphorylation (Kawai, 2001). Cyclic GMP may lower the threshold by decreasing the current threshold to generate spikes, and also prevent the saturation of odorant signals by increasing the maximum spike frequency (Kawai, 2001).

Receptor-mediated activation of phospholipase C generates IP_3, which releases calcium from endoplasmic reticulum stores by binding to IP_3 receptors .Studies in several species show IP_3 involvement in olfaction. However, electrophysiological experiments

have failed to demonstrate a specific role for IP_3 in olfactory transduction. Huque & Bruch (2004) showed phospholipase C activity in isolated catfish olfactory cilia. Bree et al (1990) demonstrated increases in IP_3 levels in response to some odorants.

Studies in primary cultures of ORNs showed that odorants stimulate the production of IP_3. Exposure of cells to low nanomolar concentrations of odorants resulted in IP_3 formation .All odorants stimulate cAMP and IP_3 production in primary culture, although with different potencies, suggesting interactions with different receptors. However, these studies were only performed at longer (1 second and beyond) times after odor encounter. Ache et al showed that odors differentially stimulate dual pathways in isolated lobster antennules .The significance of IP_3 to mammalian olfaction has been studied by knock-outs affecting the cAMP signaling cascade resulted in loss of EOG suggesting that cAMP is the only functional odorant-generated second messenger .These findings could draw a conclusion that cAMP is the primary second messenger required for the initial events of odor detection and cellular depolarization, whereas IP_3 is involved in other secondary responses, such as adaptation or activity-driven cellular responses. Whereas IP_3 is important in amphibian photo transduction, no role has thus far been found in mammals. Thus evidence of IP_3 involvement in olfactory transduction so far obtained will require further work to define their definite roles.

2.6 Other Second Messengers

The role of cGMP as primary second messenger in visual transduction is well established. cGMP may also play an important role in olfactory transduction. Odorants

increase cGMP levels in ORNs .When compared with the odorant-induced increase in cAMP and IP_3 levels, the rise in cGMP levels are slower and sustained compared to cAMP. This delayed response suggests that cGMP may not be involved in initial events of transduction, but rather in long-term effects such as adaptation. Soluble guanylyl cyclase is activated by gaseous messengers such as NO or CO, and receptor guanylyl cyclase is activated by certain odorant molecules or calcium. Both guanylyl cyclases are expressed in ORNs, implying a complex regulation of cGMP levels in olfaction (2004). Studies indicate that ORNs contain multiple cGMP pathways that mediate delayed and sustained cGMP responses to odorants.

2.7 cCAMP Transduction Cascade

Odorant molecules bind to ORs, stimulating $G_{\alpha olf}$ activation of ACIII, thereby increasing the cAMP levels, which promotes the opening of CNG cation channels (Nakamura and Gold, 1987). An influx of Na^+ and Ca^{++} ions (Frings et al., 1995; Gavazzo et al., 2000), along with Ca^+ induced efflux of Cl^- current depolarize the cilia Kleene and Gesteland, 1991; 1995; Frings et al., 2000). This membrane depolarization can be measured by electro-physiological techniques. The depolarization spreads to the dendrite electrochemically and the neuronal cell body may trigger an action potential which is conducted along the unmylenated axon to the olfactory bulb where it synapses in glomeruli and from where the odor message is carried to the cortex of the brain for perception.

2.8 G Proteins

$G_{\alpha olf}$ plays an important role in the enhancement odorant signals by coupling to AC and thereby increasing the cAMP level (Sinnarajah, 1999). It is not yet clear which components are involved or how the complex G Protein signaling system leads to enhancement, inhibition and modulation of olfactory responses. It has, however, become increasingly obvious that the Gαi plays an important role in the inhibition of AC during signal transduction. While the excitatory pathways of olfaction including $G\alpha_{olf}$ Protein mediated signal transduction is widely explored both electrophysiologically and biochemically, the inhibitory/modulation pathways including Gαi in the olfactory transduction during odor perception are yet to be explored. This study focuses on the inhibitory effect of G Protein upon coupling to odorant bound receptors during olfactory signal transduction. It is believed that Gαi protein acts on adenylyl cyclase (AC) to decrease its activity. In this study we focus on the participation of Gαi which down regulates the signal transduction through negative feed back on AC. This phenomenon is established in biochemical studies on olfactory cilia preparation (Sinnarajah, 1999).

Studies in invertebrates and vertebrates have demonstrated an important role of cAMP signaling and adenylyl cyclase (AC) activity in learning and memory. According to Pineda et a l (2004) the hippocampus, reduction of AC activity via the inhibitory $G_{\alpha i}$ is critical for memory formation, suggesting that a net balance of inhibitory and stimulatory regulators of AC is required for optimal cAMP signaling .The study shows that a selective decrease in $G_{\alpha i}$ increases cAMP signaling and enhances synaptic plasticity while impairing certain forms of memory. These findings demonstrate the existence of a tonic

inhibitory constraint applied by Gαi on cAMP production in the context of learning and memory. This study indicates $G_{\alpha i}$/AC mediated cAMP modulation in brain tissues.

It has been shown that $G_{\alpha i}$ signaling can potentiate $G_{\alpha s}$ output under certain conditions. This is mainly due to the fact that AC activity is not solely regulated by the alpha subunits released from G_i/G_o or G_q/G_{11} .In addition some AC isozymes can be regulated by Ca^{++}/Calmodulin Calcium / calmodulin dependent Kinsae. This result in stronger odor stimulus to produce sufficient cAMP to open the CNG channel, in addition to signal transduction is regulated by number of mechanisms sympathetic activation of the heart through β-adrenergic receptor mediated G_s stimulation resulted in G_s-dependent activation of AC and subsequent cyclic adenosine monophosphate (cAMP) production (Gaudin et al, 1995).

$G_{\alpha s}$ and $G_{\alpha i}/G_o$ play an important role in endocrine and metabolic functions by regulating intracellular cAMP levels. According to Song and Tao (2001) regulation of glucose metabolism by insulin may be antagonistically regulated through $G_{\alpha s}$ and $G_{\alpha i}$ mediated pathways either on the level of the insulin receptor or by influencing downstream signaling events. $G_{\alpha olf}$ is the specialized form of G_s in olfactory tissues (Herve et al., 1993 and Zhuang et al., 2000). According to Belluscio et al (1998) Electrophysiological experiments on $G_{\alpha olf}$ deficient mice exhibited remarkable reduction odorant evoked responses to all odorants tested.

Since the olfactory epithelium is continually producing neurons from a less-differentiated cell type, it is an excellent system in which to study neurogenesis, especially if it could be cultured. Ideally, primary cultures in which nerve cells are produced from their precursors as well as a method for establishing permanent cell lines

of the precursor and progeny populations. The experiments outlined here describe a primary culture system in which the formation of electrically excitable cells can be examined.

In order to establish the ability and viability of cultured ORNs for future application in olfactory research, we have attempted to obtain functional evidence that cultured OE are morphs of the acutely obtained OE and the odorant response properties of the cultured OE are identical to those of their counterparts in situ. The olfactory epithelium contains three types of cells-receptor, sustentacular, and basal which are thought to serve as a precursor population from which new olfactory neurons can arise. Isolation of receptor neurons from these other cell types has been difficult, thereby limiting ability to perform biochemical analysis. Several attempts have been made to obtain populations of primary olfactory neurons. Initial efforts employed in vitro culture of the entire olfactory epithelium (Noble et al., 1984; Gonzales et al., 1985). Hirsch and Margolis (1979) have employed enzymatic dissociation followed by general mechanical disruption, with dissociated cells centrifuged through a BSA gradient, yielding a partially purified population of cells.

Others (Calof and Chikaraishi, 1989; Pixley and Pun, 1990) have devised methods to isolate small numbers of embryonic olfactory neuronal cells for lineage analysis and electrophysiologic studies. We now describe a procedure permitting primary culture in relatively pure form of neonatal rat olfactory neurons. This technique involves enzymatic dissociation of the olfactory epithelium followed by mechanical disruption and selection of the desired neuronal cells with specific substrates and selective medium. These cells are highly responsive to low physiologic levels of odorants. The G Protein

mediated secondary transduction system serves multiple functions in the Eukaryotic organisms. Almost 80% of pharmaceutical drugs acts through G Protein receptors and will remain as the prime target in drug manufacturing. Successful mouse models of transgenic expression, including knockout strains with altered components of G-Protein system have been produced in the past to answer questions in olfaction. Mouse models have made significant contributions toward identification of the physiological role of G-Protein signaling system and thereby in the identification of drug targets. With improved culture techniques, it is possible to produce large quantities of long lived cultured OE for future applications in the field of olfactory research.

The Olfactory second messenger system discriminates among a multitude of odorant ligands of various configurations. (Lancet, D. (1986) Ann. Rev. Neurosci., 9:329-355; Snyder, S. H., Sklar, P. B. and Pevsner, J. (1988) J. Biol. Chem., 263:13971-13974).Due to this reason and because of the heterogenic nature of OE The olfactory transduction is poorly studied compared to visual , touch and taste transduction .The OE epithelium consists three principal cell types (Graziadei, P. P. C. (1971). The sustentacular or supportive cells stretch from the epithelial surface of the basal lamina, the bipolar sensory neurons dendrites extending to the surface of the epithelium and unmylenated axons through the basal lamina and the precursor basal cell, underlies the receptor neurons. Primary OE culture enriched with ORN's were used to evaluate signal transduction yielded that cAMP production to odorant stimulation with as little as 0.1 nM isobutylmethoxypyrazine (IBMP) generated a response (Ronnett et al 1993, Ronnett et al 1991). cAMP production increased in a dose dependent method with increasing odorant concentration up to a saturation point, and sometimes reappeared at still higher

(1–10 μM) concentrations. The calcium dependent signals were with maximal adenylyl cyclase activity at 10 μM free calcium and inhibition at higher calcium concentrations. The duration of the cAMP response in whole cell configuration was significantly longer than in isolated cilia.

2.9 Cultured Olfactory Tissues

In the past attempts have been made to culture primary olfactory neurons. In vitro culture of the entire olfactory epithelium did not yield viable cultures (Gonzales, et al., 1985. Noble et al 1984). Kleene and Gesteland 1981, used N-ethylmaleimide to dissociate olfactory epithelium cells into single cells, which, lost excitable properties. Hirsch and Margolis (1979) Brain employed enzymatic dissociation followed by general mechanical disruption with dissociated cells yielded a partially purified population of cells.Thus there is a need in the art for relatively pure populations of primary olfactory neurons which retain their excitability in response to odorants.

In the past OE and OB tissues been cultured with tissue explants, cellular dissociation with or without co culture (Michel,1999; Muramoto,2001; Puche,1999; Fracek,1994; Barber, 2000). Isolation of receptor neurons from these other cell types has been difficult, thereby limiting ability to perform biochemical analysis. Possibilities of studying odorant receptor expression and characteristics of olfactory receptor neurons have lead to the efforts to culture dissociated olfactory neurons (Barber, 2000).

Odorants are detectable by organisms in the micro- to nano- and picomolar range. Electrophysiological studies are conducted using millimolar levels of odorant solution (as was the case in this study), it may appear as not being in the "physiological range." However, it is not really known what the actual concentration of odorant molecules that

reaches the surface of the OE under experimental conditions. OE in situ are line with mucus that odorant binding proteins that may assist in the delivery and removal of odorant molecules to and from the receptor. Under in vitro experimental condition the mucus layer may be lost or reduced. Thus a higher stimulus intensity may be required for the stimulation and detection of odorant compounds by OE in vitro .

Past efforts to culture the olfactory epithelium and olfactory bulb have employed tissue explant, cellular dissociation, or both, with or without coculture of the complementary tissue type. Interest in developmental events, regeneration, and axon pathfinding have driven the efforts to explant epithelial and bulb tissues and grow them in culture (Farbman, 1977; Michel, 1999; Sosnowski, 1995). Interest in studying odorant receptor expression and membrane characteristics of olfactory receptor neurons has driven the efforts to culture dissociated olfactory neurons {Barber, 2000}.

An organ culture can be a relatively complex environment, compared to purified primary cell cultures or cell lines, and this complexity may mutually benefit ORNs and OBNs. ORNs survive and mature best in the presence of the olfactory bulb (Chuah, 1983; Verhaagen, 1990). Neurons, including ORNs, survive and differentiate best in the presence of astrocytes (Noble, 1984). Factors that support cell growth are likely released or expressed by other cell types in the coculture, such as olfactory supporting cells, fibroblasts, endothelial cells, and chondroblasts, as well as by the neurons themselves (Key,1996; Puche,1996; Schwob,2002).

Noble, M. (1984) have found that purified astrocytes will support the growth of olfactory epithelial neurons (OENs) in vitro.Whole-cell recordings from mitral cells (days in vitro, DIV 2–6) . To test the effect of insulin in whole-cell recordings obtained

from wt (+/+) and IR (+/−) mitral cells to determine if loss of one allele for insulin receptor would alter modulation of KV1. Decrease in peak mitral cell whole-cell current amplitude in response to loss of IR kinase could be attributed to a loss in total KV1 .

The ability of odors to evoke currents in cultured ORNs that lack processes and given the ease of patching the soma instead of the delicate cilia or dendrites (Fadool et al., 1991) and the capacity to culture ORNs (Chikaraishi 1991; Ronnnet et al., 1991 and Josephson 2004) should greatly facilitate analysis of olfactory transduction in these cells. But most of them are dissociated neurons and also used as ciliary preparations. Ronnet et al (1991) have studied electrophysiological aspects of primary cultures of purified olfactory neurons derived from neonatal olfactory tissue. For screening for odorants as well as for biochemical and physiological studies. He also suggests that neonatal tissue may be advantageous as a source material for the neuronal cultures. The cultured neurons demonstrate responsiveness to physiologic levels of odorants in vitro. He also suggested that a kit comprise the cultures of primary cultures of olfactory neurons can be used to determine the effects of odorants on olfactory neurons. The studies indicate the presence of cAMP in 5-7 DIV.

Liu N et al (1998) have studied improved method of cell culture using ZnSO4 delivery method which produces more consistent and greater yields of OE cells. Cultures established following this method contained the major cell types of intact OE and each retains characteristics found in situ. In a separate study Ronnet et al (1993) have conducted similar studies on neonatal rat olfactory neurons and proved physiologic concentrations of odorants stimulate cAMP accumulation in the cells and because of their morphology, biochemical composition, and responsiveness to odorants, these cells should

enhance olfactory investigations. They also showed that within 2 hours into plating , cells, which were initially rounded, extend processes and begin to migrate out of small clumps and within 24 hours DIV the distribution of cell were uniform and extended process with on either side one with short multi branch and other long unbranched. At 72 hours most non neural cells died off and 98% of the cells in culture were bipolar.

Cunningham et al (1999) have studied mature and immature cells in primary culture of ciliary preparation in rat pups. Cells which begin to extend processes within 24 h of plating, and by 72–96 h in culture the cells have reached maximal density. Process outgrowth was generally bipolar and in some cells were very extensive, 50–100 body lengths. By day 4 in culture, the cells were morphologically heterogeneous, but the predominant cell type (approximately 70%) was a bipolar, slightly fusiform cell with long tapering processes. The next most frequent cell type (comprising approximately 20% of the total) was a small cell with round to oval nucleus and fine bipolar processes which tended to grow in clusters. Polyclonal Golf antibody was used to identify olfactory neurons in culture, as this antibody had been shown to be a highly specific marker for ORNs in vivo D.T. Jones et al (1989).

Several in vitro approaches have been used to address both odorant-coding and neuronal development issues in the olfactory system. These include receptor transfection studies (Raming et al., 1993; Krautwurst et al., 1998), primary cultures of olfactory epithelial slices (Gong et al., 1996), and primary cultures of olfactory receptor neurons (ORNs) dissociated by enzymatic digestion of olfactory epithelia (Calof and Chikaraishi, 1989; Ronnett et al., 1991; Vargas and Lucero, 1999). However, these studies have been hampered by inefficient odorant-receptor-protein translocation to the plasma membrane

in heterologous systems (McClintock et al., 1997) or by limited survival of ORNs beyond 7 d in culture (Ronnett et al., 1991; Vargas and Lucero, 1999).

In vitro models, such as cell and organ culture, have advantages over the intact animal that make them the preferred preparation for some kinds of experiments. Physiologic experiments require the intact animal be anesthetized, which might alter cellular responses. Bleeding, respirations, or vascular pulsations can interfere with optical or electrical recordings. Anatomic investigations usually require magnification, but the tissue density of intact preparations interferes with the transillumination needed to visualize histological structures. In vitro preparations allow easier access to the system under study for making physiologic recordings, visualizing functional and morphologic changes, applying drugs and other reagents, and performing physical perturbations, such as axotomy (Suzuki, 2000).

Cultured mouse models will have significant contribution in future in the field of drug targeting, biosensor and also in identifying the physiological roles in olfaction which remains a question. We investigated the possibility of obtaining long lived culture models for electrophysiological studies, a technique that allows the measurements of electrical activity from a group of neurons as well single olfactory neuron. If an organotypic culture can be created that survives for weeks to months, then we can study with greater flexibility questions of olfactory development, the effects of odorant stimulation on olfactory function and anatomy, and the long-term effects of treatments on olfactory structures

The high level of neurogenesis in OE provides an ideal model to investigate the unanswered questions in olfactory functions. The degree of viability, competence of the

cultured ORNs and the persistence of high level of neurogenesis , survival rate and the health of our cultures supported the realization of electrophysiological experiments conducted in this study. Finally, it is worth mentioning that use of cultured OE would be important, not only to conclusively identify and characterize cultured ORNs but also to understand how they contribute to the future studies.

In summary, we are now in an era when olfactory research is fascinated by a numbers of scientists started to elucidate the multiple mechanism of olfactory perception, odor discrimination, possibility of employing olfactory cell cultures for research and identifying naval mechanisms such as odor enhancement in treating anosmia and in perfume industry. This lead to the fact that olfaction is no longer considered a matter simple sense but a complex mechanism involving factors most of them are yet to be identified, especially the early stages of the olfactory pathway. An amalgamation of biochemical, electrophysiological, genetic and behavioral methods have portrayed how odor information is processed in the olfactory system from the point of entry to the destination at olfactory cortex of the brain. From spectrum of electrophysiological approaches, we have attempted to identify mechanisms such as odorant inhibition, enhancement and also to characterize and identify the cultured ORNs as potential tissue models for futures applications in research fields such as Olfactory, pharmaceutical and Biosensors. Olfactory research has progressed rapidly in the past few decades. We have begun to glimpse at the complexity of olfaction. By providing some answers to questions in olfaction and by generating a long lived cultured models for further application of olfactory tissues in future research its not doubt that this study had made a significant contribution to olfactory research.

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3. MATERIALS AND METHODS

3.1 Chemicals:

3.1A Odorant solution (Sigma –Aldrich): A 1600 μM stock solution of the odorant mixture was prepared with de-ionized water.

3.1B Extra Cellular Fluid (ECF) (Sigma –Aldrich): Physiological buffer (Hanks balanced salt solution, modified), devoid of calcium chloride and magnesium sulfate and with sodium bicarbonate. The composition of the solution is 137 mM Nacl, 5.3 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 3.4 mM $NaHPO_4$, 5.6 mM D.Glucose, pH (7.2) Glass electrodes (7 – 10 MΩ) were filled with intracellular solution.

3.1C ICF- (Pseudo intracellular solution): Containing 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, 2 mM 3-[N-morpholino] propanesulfonic acid (MOPS) at pH 7.4.

3.1D Preimmune serum (Sigma –Aldrich).

A rabbit preimmune IgG was a kind gift of Dr. John Dennis (Auburn University). Specifications: Rabbit serum, Product number R9133, Lot: 041K9089. Amber liquid, containing 0.01% Thimerosal and protein content 50 mg/ml.

3.2 Anti $G_{\alpha s/olf}$

Effects of antibodies on the odorant-induced olfactory responses were examined by using commercial rabbit polyclonal IgG antibodies directed against $G_{\alpha S/olf}$ – subunit.

Specification: Custom order L form of antibody free of sodium azide and gelatin. Antibody is dissolved in phosphate buffered saline (PBS) Gαs/olf (C-18) L. Rabbit polyclonal IgG (Santa Cruz).

3.3 Anti $G_{\alpha i}$

Effects of antibodies on the odorant-induced olfactory responses were examined by using commercial rabbit polyclonal IgG antibodies directed against Giα-1/Giα-2 subunits.

Specification: Anti Giα-1 and Giα-2 Subunits, c-Terminal (345-354 and 346-355) (Rabbit) CALBIOCHEM. Full-line of Purified Rabbit Polyclonal Antibodies to G-Protein. The final concentration of 0.5–1 mM was obtained by diluting an aliquot of the stock solution into the pipette solution. Storage at 4^0C.

3.4 A PNC (Proteon nucleating centers)

Proteon nucleating centers (PNC) were obtained from shark blood diluted with purified water and with subsequent preparations. Shark tissue was cut into small pieces and homogenized in a blender with purified water. The homogenate was diluted in 150 ml of H_2O and centrifuged.

The supernatant was filtered through five layers of coffee filters. The filtrate was evaporated on hot plate at 95 ^{0}C and burned in crucible .The remaining ash was

suspended in 15 ml of purified water, sonicated and homogenized. The suspension was centrifuged. The supernatant was filtered successively through Millipore filters. pH of the preparation was adjusted to 7.8, sterilized in an autoclave and subsequently stored in a refrigerator.

3.4B Zinc: Indigenous products. Electro processed and chemical free, Colloidal Zinc (plus), Premium grade. 8fl.oz/240 ml.

3.4C Copper, Gold and Silver (obtained from Inspired Technologies)

Electra Clear Colloidal copper; mineral supplement; distilled water, Copper 5-10ppm

. 3.5 Preparation of Dissected Rat Olfactory Epithelium

The intact epithelial preparations were prepared following standard procedures. Rats were anesthetized by injection of pentobarbital and decapitated. The nasal septum was dissected out and olfactory epithelium attached to the septum was trimmed and kept in modified Hanks balanced salt solution (with glucose/without Ca^{++}).

3.5A Electrodes

Glass patched electrodes (World Precision Instruments, Inc., Sarasota, FL) were fashioned from borosilicate capillary tubes using Sutter puller (give the company info) to approximately -24 μm tip size for EOG and -1μm for Single cell recording.

3.5B Method

During experiments freshly dissected olfactory epithelia were placed in a buffer inside a chamber on a stage with the basal part of the tissue immersed in buffer, whereas the upper epithelial surface with olfactory cilia was exposed to the air. The chamber was connected to the head stage through a recording electrode which closed the circuit during

experiments. Odor stimuli were applied by blowing plumes of volatile odor vapors onto the surface of olfactory epithelium. The pressure and duration of the application is controlled by a PV 800 pneumatic Pico pump which uses dried and charcoal filtered nitrogen gas to propel the odor molecules from the container.

After the application of the odor, lingering odor molecules are removed from the vicinity of the tissue by a weak vacuum regulated by the same instrument. Rat septal olfactory mucosa were dissected out and placed in a perfusion chamber such that the basal portions were immersed in physiological buffer (containing 137 mM NaCl, 5.3 mM KCl, 4.2 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 3.4 mM Na_2HPO_4, and 5.6 mM D-Glucose at pH 7.4), while the epithelial surface with olfactory cilia were exposed to air. Patch electrodes of approximately 7 MΩ were filled with ICF then connected to a patch-clamp amplifier in order to detect responses from the olfactory epithelium.

By carefully approaching the cilia of olfactory sensory neurons with a fine recording glass pipette, it was possible to obtain electroolfactogram recordings. Downward responses indicate inward current. Once contact between the electrode and the surface of

The olfactory epithelium was formed, air puffs of odorant mixture containing 1.6 mM ethyl butyrate, eugenol, and (+) and (–) carvone were applied. Odor responses over the time course of fifteen minutes were recorded after being amplified (Gain=1) by a patch-clamp amplifier and filtered at 2 - 5 kHz. Data were digitized using digidata 1322A (Axon instruments).

Odor is applied through a small Teflon nozzle that had been positioned in the direction of the tissue. A multibarrel pipette placed downstream from the recording site, was used to deliver odorant stimuli by pressure ejection through a Pico pump. Odorant solution was prepared as 1600 μm stock in deionized water to the final volume of 500 ml solution. Electrical responses propagated by the application of odor are detected and amplified by the amplifier instrument. Data are relayed from the amplifier to the computer through the interface hardware. The responses are then processed and plotted by the computer for real time viewing, as well as for storage and subsequent analysis.

3.6 Olfactory epithelium _Olfactory bulb culture:

Using the roller tube method of culturing, epithelium-bulb co-cultures, explanted from 1-4-day-old Swiss Webster mice were allowed to grow on Aclar cover slips for periods ranging from 18 hr to 36 days. The explants flattened so that in some areas, the culture was only a few cells thick, making individual cells distinguishable with magnification.

3.6A Preparation of cultured mouse olfactory epithelium

Post natal day (PND 1-4) Mouse pups were anesthetized by halothane inhalation and decapitated mid-sagittally. Split heads were put in dissecting buffer (Hank's balanced salt solution, D-glucose 10 mg/ml, cefazolin 16 μg/ml). Olfactory epithelium was elevated from cartilaginous septum and olfactory bulb was dissected from cerebrum and cribriform plate (olfactory nerve axons were severed during the process of dissociation). Tissue was left in fresh dissecting buffer for about 1 hour. Film of chicken plasma was applied to Aclar plastic strip and explants were placed on strip, in contact with each other. A drop of bovine thrombin was added to form a clot over explants. The strip was

placed in 15 mm culture tubes with 1-2 ml of growth medium (Eagle's Basal Medium, 68% Earle's balanced salt solution, 25% fetal bovine serum, 7% insulin, 5 μg/m glutamine, 2 mM D-glucose, 16μg/ml, ± nerve growth factor, 10 ng/ml).

Tubes with cultures were placed in roller drum set to rotate ~15-20 revolutions/hr in a incubator at 37 ^{o}C. Growth medium was changed once a week. To provide a reference for comparison, EOG responses were recorded from dissected rat olfactory epithelium. The head was split mid-sagittally and the nasal septum and turbinates were exposed. The turbinate OE was left attached to the underlying cartilage whereas the septal OE was pulled away from the cartilage. EOG recordings were carried out in a perfusion chamber containing HBSS (137 mM NaCl, 5.3 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 3.4 mM Na_2HPO_4, and 5.6 mM D-glucose at pH 7.4) with calcium and magnesium. The EOG set-up and odorant mixture solution were the same as those used for the cultures.

3.7 EOG experiments

Rat or dog olfactory epithelia dissected out immediately after decapitation were placed in Hank's balanced salt solution (with glucose). The tissue is then transferred to a temperature regulated chamber filled with the same buffer.

Using a 3-dimensional micromanipulator, a glass patch-electrode (0.5 MΩ resistance), which was filled with ECF, metal solutions such as Zn, Cu, Au, Ag and PNC were secured in an electrode holder, was lowered onto the surface of the olfactory epithelium until it made contact with the surface.

At this point, the tissue was electrically connected to the amplifier through the ground electrode and the recording electrode (inside the glass patch-electrode). Odor is applied

Through a small Teflon nozzle that has been aimed in the direction of the tissue. Dose dependency EOG measurements were obtained by the incorporation of the metal nanoparticles with the odorants of interest and delivery of the odorant /metal nanoparticle mixture to the odor receptors. Its was found that the odorant/metal nanoparticle mixture can be delivered out of an aqueous media and result in significant stimulation of the olfactory neurons as measured by EOG.

3.8 Single cell recording

Rat olfactory epithelium was placed in modified Hanks balanced salt solution without Calcium. The tissue is then transferred to chamber filled with the same buffer. A glass recording electrode (~ 7 mΩ/Tip size ~ - 1μm), which was filled with ICF for control experiments and subsequently with PNC, Zn, Cu, Au, and Ag solutions and secured in an electrode holder, is lowered Using a three dimensional micro manipulator, onto the surface of the olfactory epithelium in order to obtain cellular contact. At this point the tissue is electrically connected to the amplifier through the ground electrode and the recording electrode which is connected to the head stage by means of a holder and will make contact with the solution inside the glass recording electrode. Single cell configuration also yielded dose dependency with incorporation of the metal nanoparticles with the odorants of interest and delivery of the odorant /metal nanoparticle mixture to the odor receptors as of EOG.

3.9 Protocol:

3.9A EOG

Voltage clamp recordings were made for 15 minutes with the introduction of Odorant puffs as stimulus. Each stimulus lasted for 0.5 seconds and the voltage fluctuated between 63 mV to 70 mV. By carefully approaching olfactory sensory neurons with a fine recording glass pipette, it was possible to obtain Single-cell voltage-clamp recordings. Recordings were made after stable contact with the olfactory neuron. Stable electrical recordings were obtained from dissected olfactory epithelia. The mean value for the resting potential, V_o, was -70 ± 3.6 mV and ranged between –70 to -63 mV. Downward responses indicate inward current.

3.9B HFOS

High Frequency Odorant Stimulation (HFOS) was applied to cultured mouse OE and rapidly isolated rat, cat and dog OE. Adaptation EOG responses were examined after the application of stimulations of high frequency with increasing inter stimulus intervals and sustained odorant stimulation. OE was subjected to inter stimulus intervals between 200 ms to 20 seconds and with sustained stimulation lasting up to 20 seconds.

3.9C Single cell recording

Current clamp recordings were made with the introduction of odorant puffs as stimulus. Recordings were done for 15 minutes with 1 minute inter-stimulus intervals in the case of experiments with antibodies against G Protein and metals. Each stimulus lasted for 0.5 seconds.

Currents were measured with the Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) in the single-cell voltage-clamp mode. Patch pipettes were fabricated using borosilicate capillaries (WPI, Sarasota, FL) and pulled using a P-87 pipette puller (Sutter Instruments, Novato, CA). Pipette resistances were 5- 10 MΩ when filled with the internal solution. Voltage-clamped currents were low-pass filtered at 10 kHz.

Electrical responses propagated by the application of odor were detected and amplified, the data were relayed from the amplifier to the computer through an analog-to-digital interface Digidata 1322A (Axon Instruments). Acquisition, storage and analysis of data were performed using PClamp 9 software (Axon Instruments). Resting membrane potential was measured as the potential at which the current was zero. Experiments were performed at room temperature.

3.9D Data Analysis

The voltage signal of the EOG responses digitized and stored as a data file in the computer. The voltage signal was filtered at 0.1-1 kHz, to eliminate unwanted noises. Clampfit software was used to analyze the half rise time (½ time to peak) decay time (½ time to decay from peak amplitude and the width of response (Time between rise and decay) of the EOG responses.

Odor responses over the time course of several minutes were amplified by a MultiClamp 700A patch-clamp amplifier (Axon Instruments Inc., Union City, CA) and analyzed by the Clampfit data analysis software (Axon Instruments).

Clampfit software was used to analyze the EOG and to derive values for the Rise time (R T ½), decay time (D T ½) and width (width at ½ rise and decay ½) of the EOG responses.

Statistical analysis was done on EOG responses from cultured OE. All values are expressed as mean (±) S.E. Differences between groups were examined for statistical significance using t-test. The variables in EOG response of cultured mouse OE (amplitude, rise time, decay time and width) were measured. A significant change was observed by comparing the decay time between the two age groups at ½ T Decay time. Values in the text are means (±) S.E. For analysis, 8-10 consecutive traces were averaged. Results are expressed as mean (±) SE.

4. ELECTROPHYSIOLOGICAL VIABILITY STUDIES OF OMP – POSITIVE MOUSE OLFACTORY RECEPTOR NEURONS IN VITRO

4.1 Abstract

In order to compare and contrast the electrophysiological characters of cultured olfactory epithelium (OE) to that of rapidly isolated OE, EOG responses were obtained from olfactory receptor neurons in organotype olfactory epithelium (OE)-olfactory bulb (OB) cultures at various stages and protocols. In a parallel study, fluorescence immunohistochemistry and confocal microscopy were employed to characterize the cellular populations of the same cultures (Josephson et al 2004). The cultures were exposed to charcoal-filtered air, individual odorants, and a mixture of (+) and (-) carvone, eugenol, and ethyl butyrate following which the EOG potentials were recorded. Another embodiment of the study was to subject the cultured neurons with varying inter pulse periods to explore the system's ability to respond to and adapt to odorant exposure. EOG responses of cultured OE were similar to those of rapidly isolated OE. Cultures 3-4 days old (n = 8) showed rapid rise times but prolonged decay times. Cultures 13-15 days old (n = 8) showed both rapid rise and decay times.

The cultures contained cells immunopositive for olfactory marker protein, a protein found in mature olfactory receptor neurons in olfactory epithelium in situ and other cells positive for b-tubulin, a protein found in maturing neurons (Josephson et al 2004). These EOG results extend the characterization of our olfactory epithelium-olfactory bulb organotypic culture system and suggest that olfactory epithelium passes through an immature stage in the week following explantation and culturing. This likely reflects on-going development of olfactory receptor neurons that were immature, or not yet born, at the time of culturing.

Although all cultures responded to the four individual odorants in equimolar concentrations, their response to each odorant was variable with ethyl butyrate showing the strongest response on average. At high frequency stimulation with an interstimulus interval (ISI) of 200 ms, the EOG responses appeared to be summated. With increased interstimulus intervals, adaptation was clearly apparent with partial recovery of the second and succeeding responses. At ISI of 800 ms, adaptation occurred with the second and succeeding responses of lower amplitude than the first. This trend continued and the membrane potential remained depolarized until the duration of the inter-stimuli intervals reached 20 seconds, where the second and succeeding response amplitudes were equal to that of the first EOG amplitude.

The cultures contained cells immunopositive for olfactory marker protein, a protein found in mature olfactory receptor neurons in olfactory epithelium in situ and other cells positive for b-tubulin, a protein found in maturing neurons (Josephson et al 2004).

These results extend the characterization of our olfactory epithelium-olfactory bulb organotypic culture system and suggest that olfactory epithelium passes through an immature stage in the week following explantation and culturing. This likely reflects on-going development of olfactory receptor neurons that were immature, or not yet born, at the time of culturing.

The survival rate and the health of our cultures supported the realization of electrophysiological experiments conducted in this study. Cultured OE is odorant responsive from day one. The cultured neurons exhibited typical EOG responses and were resilient during intense and prolonged experiments. This indicates the feasibility of using in vitro OE's. Olfactory receptor neurons as old as 100 DIV responded to odorants. The main factor known to enhance ORN survival include physical contact with olfactory bulb tissue. We show that long-term culture of OE and OB tissues is possible and it is a promising model for future electrophysiological studies and most importantly in biosensor applications using membrane receptors as biosensors.

4.2 Materials and Methods

The critical methods are described in detail. Some of the described methods were routinely used in our lab.

4.2 A Solutions and reagents

4.2 A1 ECF - Physiological buffer (Modified Hanks balanced salt solution): With sodium bicarbonate without phenol red, calcium chloride and magnesium sulfate - containing 137mM NaCl, 5.3mM KCL, 0.44 mM KH_2PO_4, 4.2mM $NaHCO_3$, 3.4 mM $NaHPO_4$, 5.6 mM D Glucose, pH 7.2).

4.2 A2 ICF (Pseudo intracellular solution): Containing 110mM KCL, 4mM NaCl, 2mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, 2 mM MOPS at pH 7.4.

4.2 A3 Chemicals Odorant solution (Sigma –Aldrich) A 1600 μM stock solution of the odorant mixture was prepared with de-ionized water containing 1600μM in 1 L:0.0016 moles of -Carvone, + Carvone, Eugenol and Ethyl butyrate.

4.2 B Animal Tissues

4.2 B1 OE-OB Cultures

In order to explore the feasibility of using cultured mouse OE as a model for future olfactory research, OE from neonatal explants of Swiss Webster mouse olfactory epithelium was grown alongside of OB was selected as described in Josephson et al (2004).

Adult mice were allowed to deliver their pups in their home cages. Neonatal pups were anesthetized by halothane and decapitated. Olfactory bulb and epithelium were dissected out, rinsed and placed on an Aclar cover slip in a culture tube allowing a growth of a complex organ culture of olfactory tissue.

Using the roller tube method of culturing, epithelium-bulb co-cultures, explanted from 1-4-day-old Swiss Webster mice were allowed to grow on Aclar plates for periods ranging from 18 hr to 95 days. The OB explants flattened so that in some areas, the culture was only a few cells thick, making individual cells distinguishable. The OE explants retained much of their original shape and thickness.

4.2 B 2 Acutely dissected OE

The rapidly isolated Sprague Dawley rats OE were obtained by the following standards methods. Rats were anesthetized by injection of pentobarbital and decapitated. The nasal septum was dissected out and olfactory epithelium attached to the septum was trimmed and kept in modified Hanks balanced salt solution (with glucose/without Ca++).

4.2 C Electrophysiological Recording

4.2 C1 Electroolfatography

Fragments of acutely dissected olfactory mucosa whole OE-OB cultures were placed in a recording chamber such that the basal portions were immersed in physiological buffer solution, while the upper epithelial surface with olfactory cilia was exposed to the air. A minimum of 4 replications were performed.

The parameters of EOG kinetics such as rise time, decay time, width across the arms of rise and decay and EOG amplitude were calculated.

Using a 3-dimensional micromanipulator, a borosilicate glass patch-electrode (24 μm tip diameter, 0.5 MΩ resistance), filled with Hank's balanced salt solution and secured in an electrode holder was lowered onto the surface of the olfactory epithelium until it made contact with the surface. Once contact between the electrode and the surface of the olfactory epithelium was made, air puffs of odorant mixture containing 16M ethyl butyrate, eugenol, and (+) and (–) carvone were applied.

Odor was applied through a small Teflon nozzle (a multibarrel pipette) positioned in the direction of the tissue, downstream from the recording site, by pressure ejection through a Pico pump.

Odor activated current clamped voltage responses were recorded in the form of EOG with an integrating patch clamp amplifier (Axon 700A). The analog signal was filtered at 2 - 5 kHz and digitized using a digital converter. Data acquisition and subsequent storage and analysis of the data were carried out using pCLAMP software (Axon Instruments).

We exposed organotypic olfactory epithelium-olfactory bulb cultures of different ages to charcoal-filtered air, and the odorants (+) carvone, (-) carvone, eugenol, and ethyl butyrate, either individually or as a mixture, at varying concentrations or with varying inter pulse periods to explore the system's ability to respond to and adapt to odorant presentation.

Culture tissues from 1 DIV through 100 DIV were tested for odor responsiveness and compared with that of typical EOG's obtained from rapidly isolated rat OE tissues. EOG kinetic variation with odorant concentration, individual odorant application and adaptation responses were carried out in each culture, and the EOG response patterns were compared to typical EOG response from their actually collected counterparts in situ. Statistical analysis was performed using results of within and between the culture groups in order to draw conclusions.

The variables in EOG response (amplitude, rise time, decay time and width) of cultured mouse OE at different in vitro ages to different odorant concentrations, or to sustained odorant stimulation were measured at half time. Clampfit software was used to

analyze the EOG and to derive values for the Rise time (R T ½), decay time (D T ½) and width (width at ½ rise and decay T) of the EOG responses.

Odorant solutions ranging from 1 to 16 M were delivered to the cultured OE using the Pico pump at 5 Psi, 0.5 Sec. Puffs of charcoal filtered air were delivered in between the odorant puffs to ensure removal of any lingering odorant. The interval between puffs was 1 minute. Ethyl butyrate, eugenol, (+) carvone, and (-) carvone were also delivered as single odorants at 16 M concentration using the same delivery parameters used to deliver the mixture.

Adaptation study involved a series of High Frequency Odorant Stimulation (HFOS) EOG recordings of cultured OE and acutely dissected OE from rat, dog and cat with sustained odorant stimulation and with an increasing frequency of odorant stimulation.

Experiments were conducted using 16 M concentration of odorant delivered with15 Psi pressure. HFOS with time intervals between stimuli of 200 ms, 800 ms, 4000 ms and 20 sec were applied.

4.2 C2 Whole cell recording

We recorded current changes from a soma of an individual OE neuron in culture in response to puffs of odorant mixture. The odorant mixture was delivered at 1 minute intervals for 0.5 sec (stimulus duration) over a 15 minute recording period. Measurements of the response of OE to odorant mixture were taken in the single-cell configuration, voltage clamped at 68 mV.

4.3 Results and Discussion

4.3 A Results

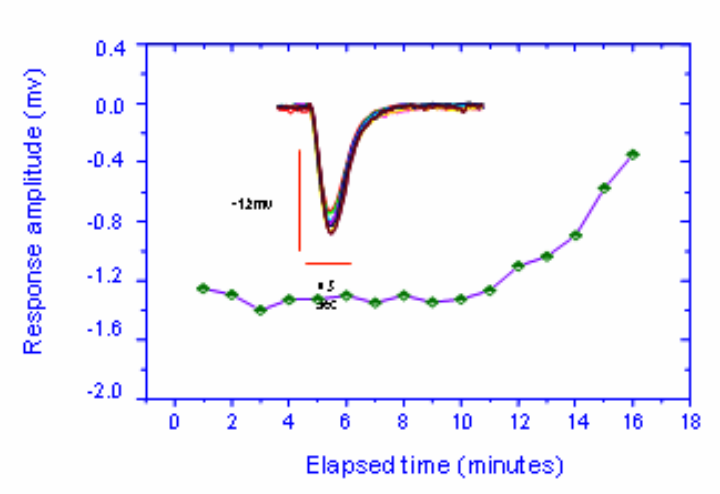
EOGs and whole cell odorant responses from in vitro olfactory cultures have not been previously published except in preliminary form (Josephson et al 2004).The present study established the possibility of stable electrical recordings from OE_OB Co cultures in the form of EOG and whole cell methods. They are also the first to measure odorant responses from organotypic culture system to different odor concentration, variance in response to individual odorants, adaptation patterns and single cell responses.

In cultures that responded, puffs of vapor from an odorant mixture induced small positive and dominant negative EOG potentials that were synchronized with the onset of the stimulus (figure 1). Stimulation of charcoal-purified air alone did not yield any responses, nor did stimulation of odorant vapor over areas of bulbar cells.

4.3 A2 Electro-Olfactogram

We recorded EOG responses from ages 1 to 100 DIV. In each age group more 85% of the EOG recordings were successful. EOG recordings obtained from olfactory epithelia. The mean value for the resting potential, V_o, was -70 ± 3.6 mV.

Figure 1

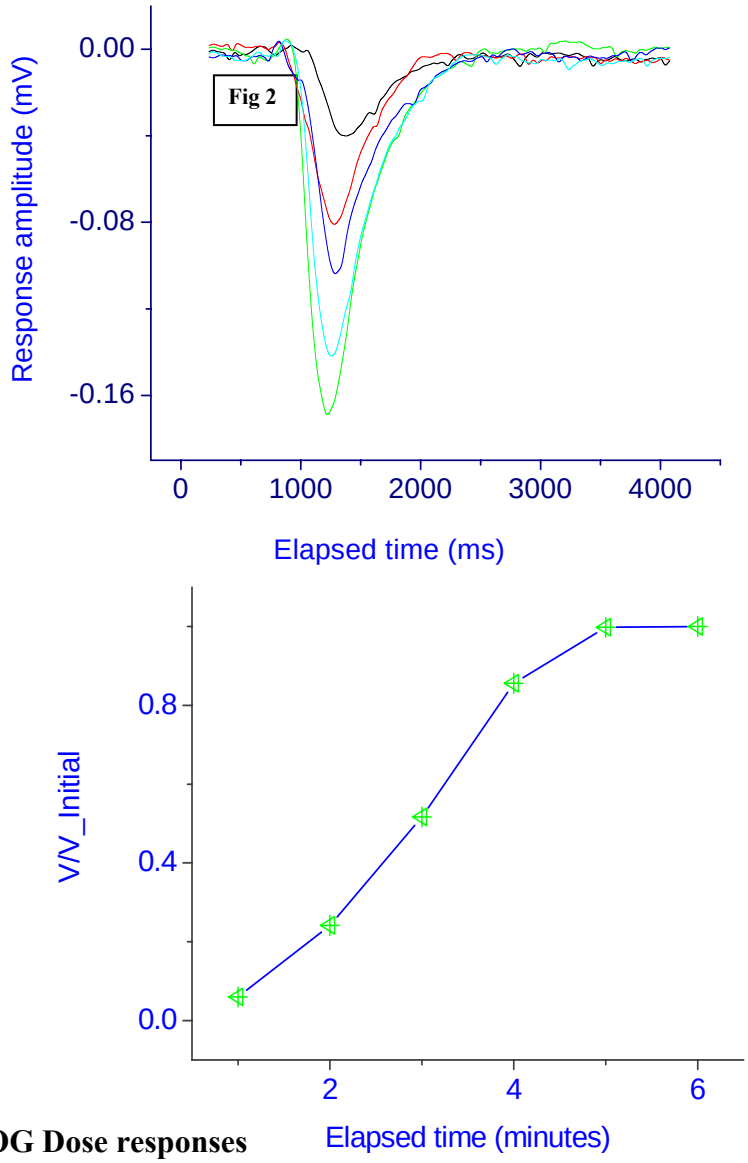


Plot of odorant induced EOG amplitude versus elapsed time in cultured OE. Odorant pulses were with 1 minute inter- pulse intervals and for 15 minutes period. (Insert) Real time EOG recording of odorant-evoked EOG responses in cultured OE upon perfusion of ECF through glass electrode.

Cultured OE responds with an initial electropositive (upward response) followed by electronegative (downward response) EOG when exposed to a 0.5-sec puff of vapor from a 16 mM odorant mixture solution (OE + odor). Electronegative EOGs were not observed when the same region of the culture was exposed to puffs of charcoal-purified air (OE + air). The time of application for the 0.5-sec odor/air puff is indicated by the time line marker.

Odorant solutions ranging from 1 to 16 M was delivered using the Pico pump at 5 PSI and odorant width of 0.5 S was applied to the cultured OE. Puffs of charcoal filtered air were delivered in between the odorant puffs to ensure removal of any lingering odorant.

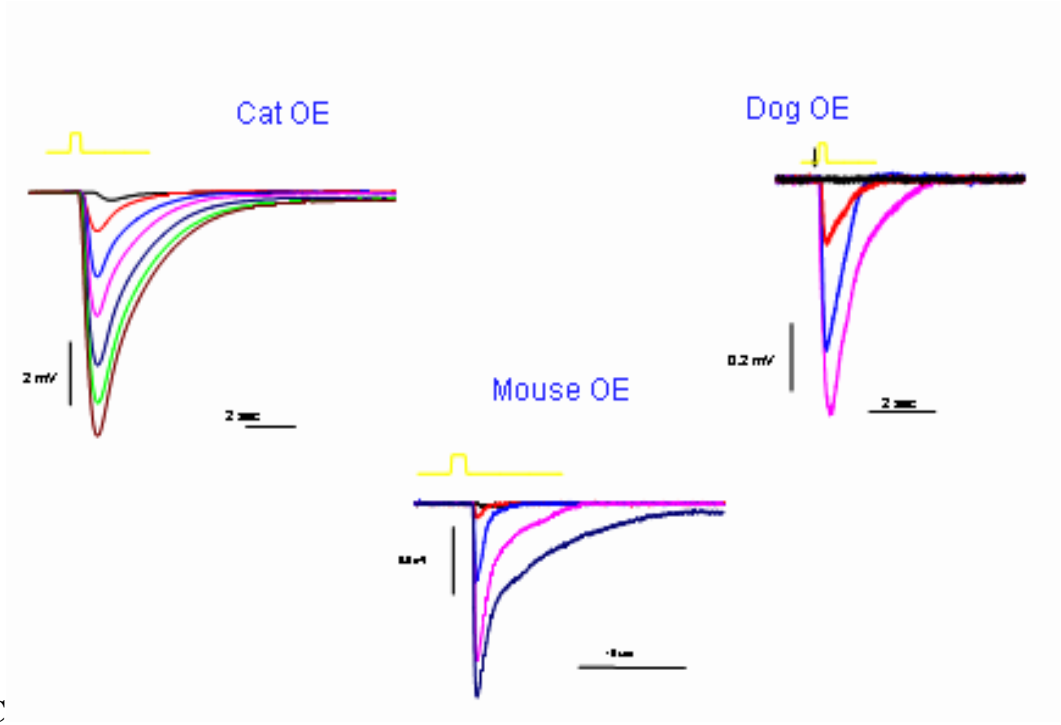
The EOG responses recorded are from the same tissue at the same site with an interval of 1 minute between puffs. (Fig 2). This dose response pattern resembles dose responses obtained from rapidly isolated cat, dog and rat EOG (figs 3a, b, and c respectively).



A 3 EOG Dose responses

Fig 3

A **B**



C

Rapidly isolated OE were exposed to 100,200,400, 800 and 1600µM odorant mixture and dose response showed increased amplitude with increasing odor concentration. These traces are from one tissue and were recorded from the same site on the olfactory epithelium.

This relationship was also true for each of the tissue in the same culture tested and for the averaged response. In addition, dissected rat OE, dissected mouse OE, dissected cat OE and dog OE exhibited a similar pattern of dose response EOG with different EOG amplitude. The EOG response amplitudes are presented as a v/v_ initial for comparison.

A4. Rise, decay time

The ½ rise time (Fig 4) ranged from 260.87 ms to 409.47 ms [mean=341 ms] in 5 DIV and 207 ms to 474 ms [mean=277] in 13 DIV, the ½ decay time ranged from 996.21 ms to 1483.24 ms [mean = 1239.44 ms] in 5 DIV and 339.137 ms to 553.53 ms [mean = 438 ms] in 13 DIV and the half width time ranged from 1249.95 ms to 2185.5 ms [mean=1723.32 ms] in 5 DIV and 511.87 ms 819.46 ms [mean=595.00] in 13 DIV, whereas the EOG amplitudes for both age groups ranged from 0.05 –0.08mV [mean = 0.065 mV].There were no significant EOG responses to the puffs of air or OB

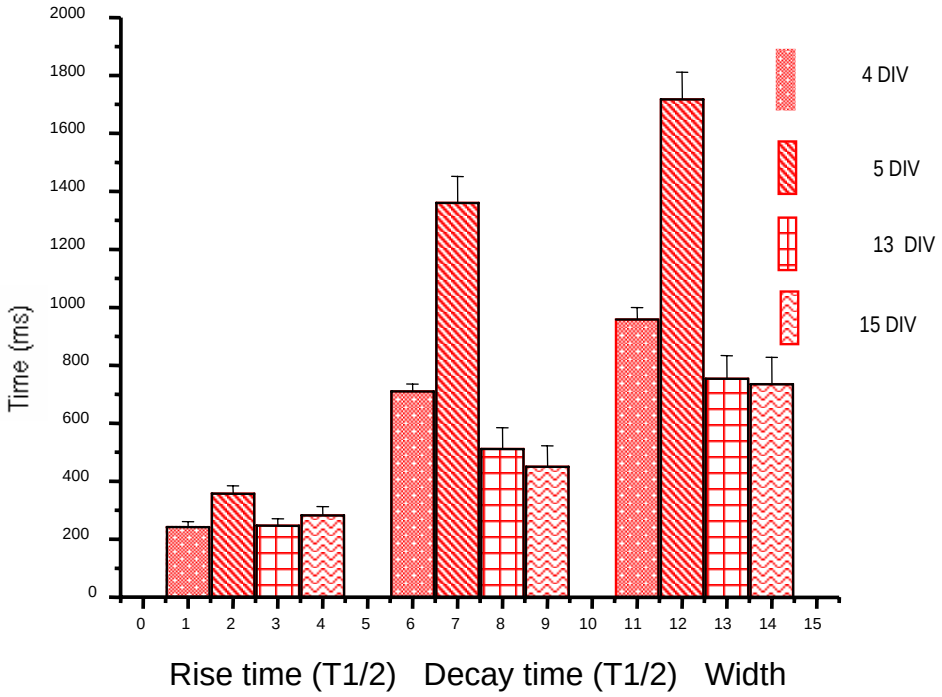


Fig. 4. Changes in EOG kinetics between different age groups of cultured OE Stimulation of 4, 5, 13 and 15 DIV cultures with odorant vapor reveals that they'r sensitive to odor stimuli in vitro and posse's characteristic response pattern witl regards to decay time.

The EOG response of 5 DIV and 13 DIV Cultured mouse OE is illustrated above. In both cases, the EOG response was characterized by a similar response amplitude and a rapid rise to peak (negative potential) .The primary differences between the EOG response in 5DIV and 13 DIV cultures were the comparatively faster decay rate in 13DIV and a much slower return to the baseline in 5 DIV which corresponded with the width at ½ t between the two age groups.

Figure 5 a, b, c, d, e and f show the EOG amplitude response from different age groups of cultures.

Fig 5 a
3 DIV

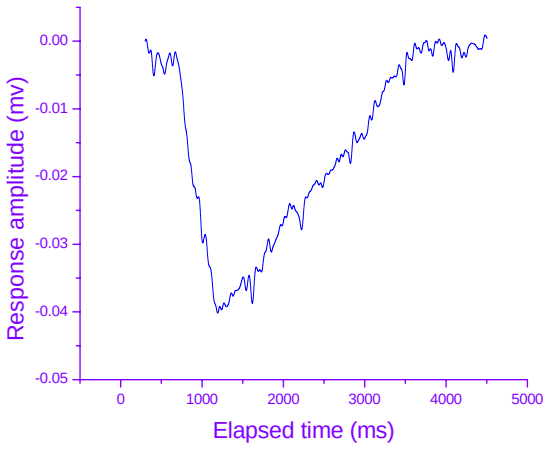


Fig 5 b
5 DIV

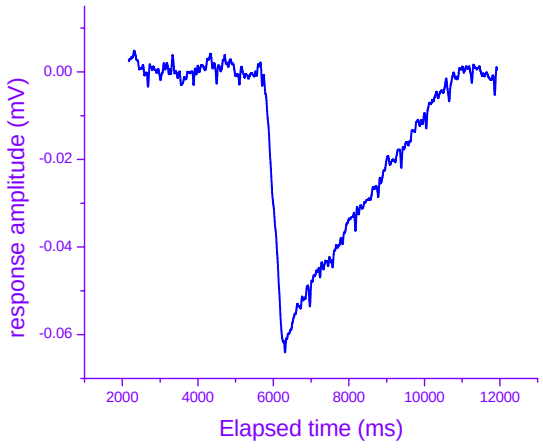


Fig 5 C
7 DIV

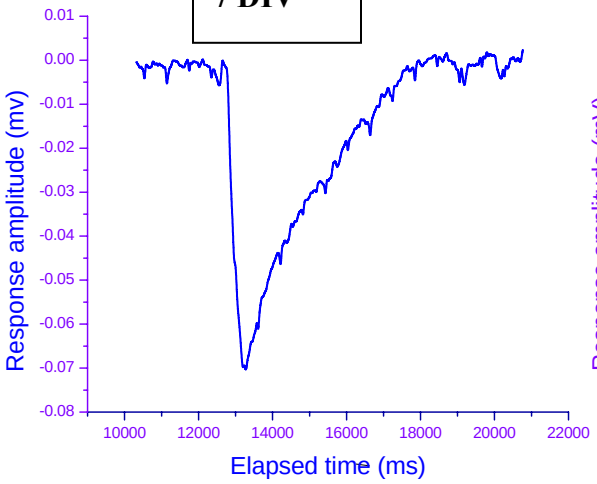


Fig 5 D
13 DIV

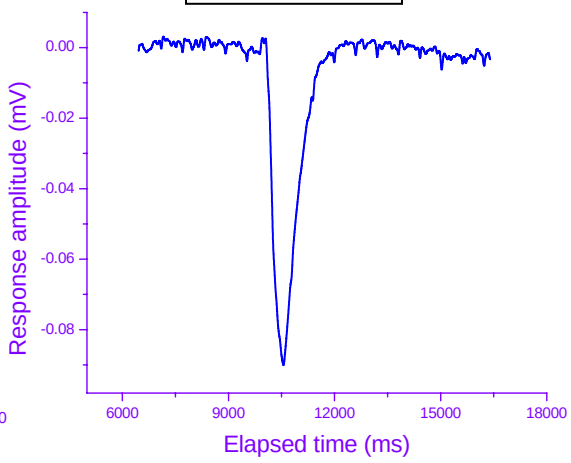


Fig 5 E
15 DIV

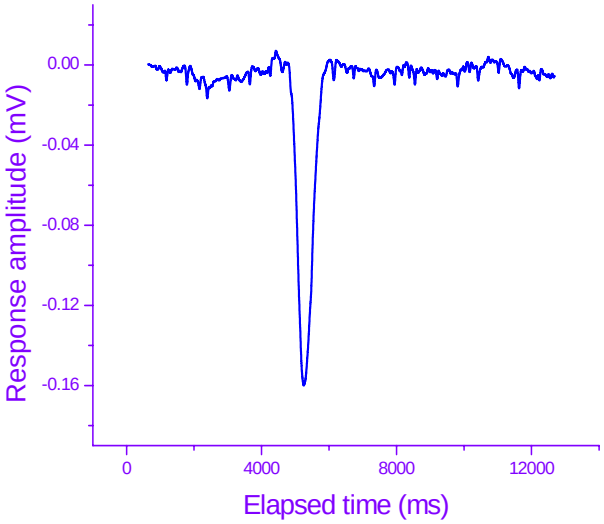
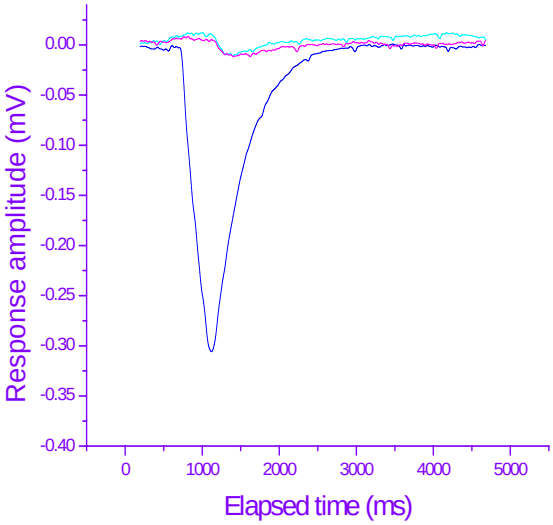


Fig 5 F
36 DIV



The decay time of the EOG response was significantly varied in 5 DIV when compared to 13 DIV. The mean decay time in 5 DIV was 1239.44 ms+/- 148 and 438.46+/- 62 In 13 DIV. The ½ width time of the EOG response corresponded with the differences in decay time, where 5 DIV WAS 1723.32+/- 280 AND 13div was 595.00 +/- 58

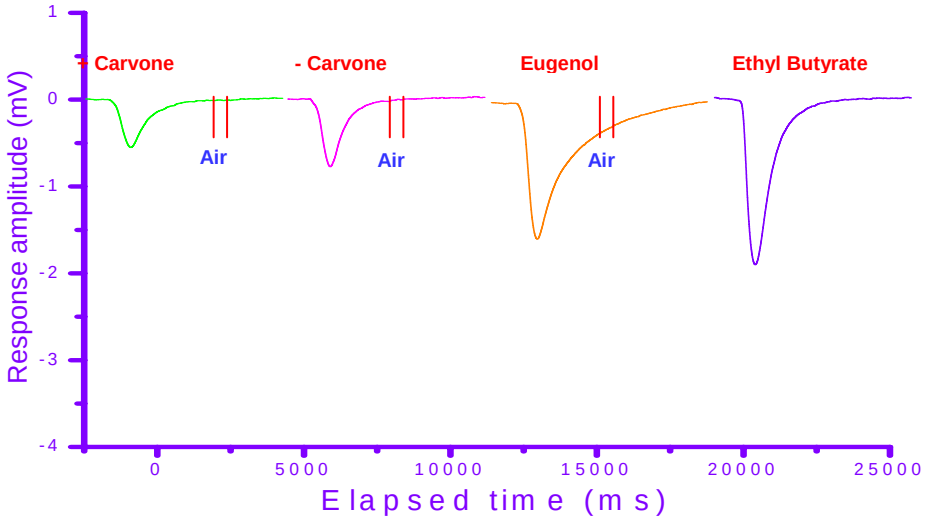
However the rise time of the EOG response did not yield any significant variation between the age groups.

The rise time, decay time and width time of the EOG response amplitudes are presented as ½ of the EOG amplitude (for rise, from half to peak amplitude and for decay from peak to half amplitude towards baseline) The ½ rise t, ½ decay time and the ½ width time of the odorant puff was calculated for each age group and for each tissue.
In 5 DIV the mean decay time was1239.44 ms+/- 148 , whereas the mean decay time for 13 DIV was 450.46+/- 62 and in 5 DIV the mean width was 1723.32+/- 280 whereas the mean decay time for 13 DIV was 595.00 +/- 58. These differences are statistically significant. The mean amplitude remained similar in all the EOG amplitude response.

A5 Individual Odorant puffs

Both cultured OE and dissected rat olfactory epithelium were subjected to puffs of individual odorants from the odorant solution. Ethyl butyrate caused the maximum amplitude when compared to other 3 odorants which followed the same pattern (figure 6).

Figure 6



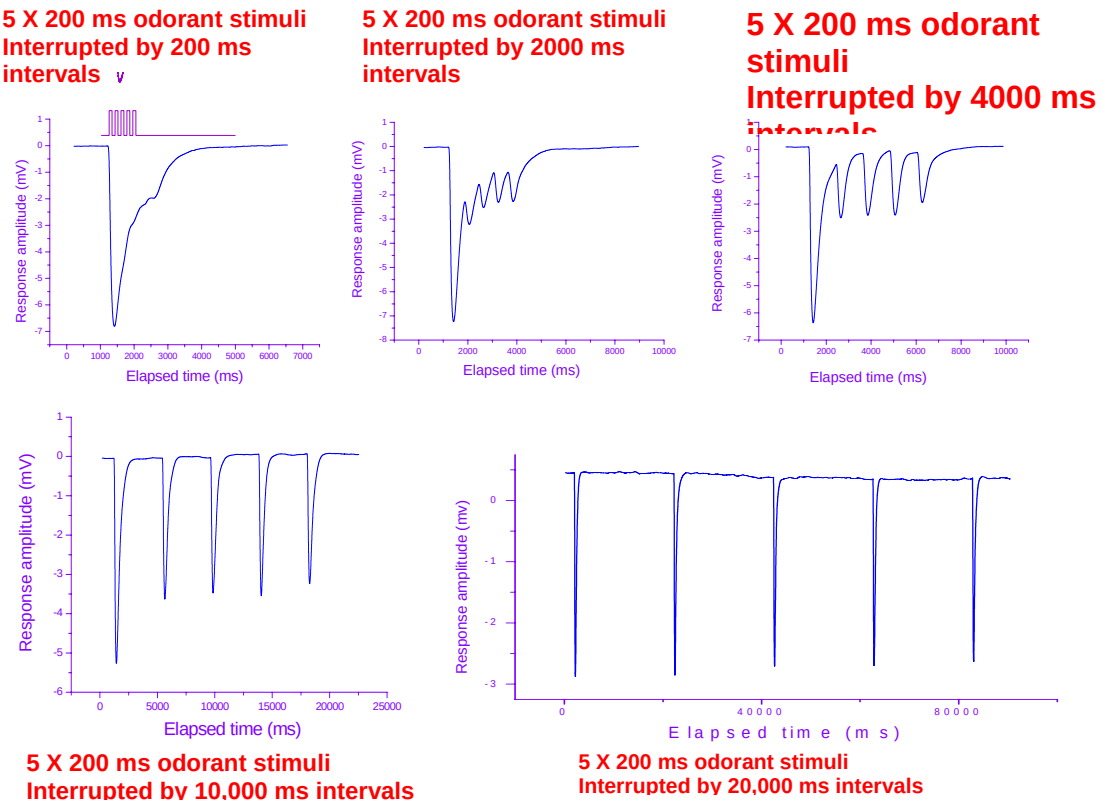
EOG recording from cultured OE to individual odorants. The recordings were obtained from the same site of OE. Interval between Inter-pulse was 1 minute Two puffs of air in-between the odorant pulses was given to drive off any lingering odor from the ciliary region.

A6 Adaptation Study in 13 DIV mouse OE

At high frequency stimulation with an inter-stimulus inter-stimulus (ISI) of 200 ms, the EOG responses appeared to be summated. With increased ISI, adaptation was clearly apparent with partial recovery of the second and succeeding responses. At an ISI of 800 ms, adaptation occurred with the second and succeeding responses of lower amplitude than the first. This trend continued and the membrane potential remained depolarized until the duration of the ISI reached 20 seconds, where the second and succeeding response amplitudes were equal to that of the first EOG amplitude. (Figure 7).

Adaptation EOG kinetics of a 13 DIV mouse showing odorant induced olfactory responses where an increase in interstimulus intervals resulted in complete recovery of odorant response.

Figure 7



4.3 B Discussion

Olfactory epithelium consist of three types of cells namely, receptor, sustentacular cells and basal cells (Morrison E.E 1991). The basal cells give rise to new olfactory neurons as the old neurons wither off. This unique nature of neurogenesis in ORNs leads to the development of several culture methods to study their properties in vitro.

An important step in establishing and utilizing a cell culture system for the in vitro study of olfaction is assessing whether the cultured cells possess physiological properties similar to those of acutely obtained olfactory neurons. Various investigators have successfully established proliferating cell lines from olfactory tissue, but few have demonstrated the characteristics of odor sensitivity of these cells. This method will establish a successful cultured cell lines from neonatal olfactory tissue and will demonstrate their ability to respond to odor stimulation using EOG and single cell recording techniques.

Biochemical studies in the past have established the olfactory nature of these cells by the presence of three olfactory-specific markers. Immunoreactivity for $G_{\alpha olf}$ and ACIII, two components of the olfactory second messenger cascade (Bakalyar and Reed 1990), could be observed in both permissive and nonpermissive conditions. Primary cultures of purified olfactory neuron indicate the presence of cAMP in 5-7 DIV.

Biochemical studies requiring isolated olfactory neurons from the epithelium have been difficult due to practical difficulties of isolating single neurons. In the past, several studies have been conducted to obtain populations of primary olfactory neurons.

A pioneer studies employed in vitro culture of the olfactory epithelium as a whole (Noble et al., 1984; Gonzales et al., 1985). Methods using N-ethylmaleimide to dissociate olfactory epithelium cells into single cells, resulted in, lose of excitable properties of the neurons (Kleene and Gesteland, 1981). A study by Hirsch and Margolis (1979) employed enzymatic dissociation of olfactory tissue followed by general mechanical disruption.

Calof and Chikaraishi, 1989; Pixley and Pun, 1990 employed methods to isolate prenatal olfactory neurons for lineage analysis and electrophysiological studies. However, these studies have been hampered by limited survival of ORNs beyond 7 days in culture.

To overcome the survival issue, there have been several attempts to develop "immortal" olfactory cell lines (Ronnet et al 2001). Another technique involved a procedure obtaining primary culture in relatively pure form of neonatal rat olfactory neurons with enzymatic dissociation of the olfactory epithelium followed by mechanical disruption and selection of the desired neuronal cells. These cells were highly responsive to low physiologic levels of odorants. Liu N et al (1998) have studied improved method of cell culture using ZnSO4 delivery method which produces more consistent and greater yields of OE cells. Cultures established following this method contained the major cell types of intact OE and each retains characteristics found in situ.

Olfactory neurons have been cultured as an in vitro models for studies in olfactory development (Farbman, 1999) the process of cell maturation and integration into the olfactory bulb [Josephson et al 2004, Liu. N et al 1998], and the ability of cultured OE to respond to odorants.

We hypothesized that a co culture of OB and OE will be an ideal culture model to establish a long-lived culture system that includes mature olfactory receptor neurons in synaptic contact with olfactory bulb neurons. By growing the initial olfactory synapse in culture, we hope to create a readily accessible in vitro system that would allow us to study how odorant signals are regulated, what effect these signals have on receptor neuron connections with bulbar neurons, and how maturing receptor neurons find their synaptic target.

A typical EOG response curve will have a rapid rise with latency time and a comparatively slower decay towards the baseline. The decay may accompanied by a plateau, if the stimulus time is more than required for a typical depolarization pattern of the neuron.

The EOG response recordings obtained from our cultured tissues fitted the characteristic features of a typical EOG. The magnitude of EOG which is partly dependent on the number of cilia, the nature of tissue and number of channels expressed in cilia. However in our present study the average magnitude of EOG responses were comparable to that of acutely obtained OE. In the present studies we did not see a pattern in the magnitude of EOG in different age groups of OE. Process formation (number of cilia) and development in culture is expected to have an influence on the EOG amplitude. Ottoson (1956) and Coates (1990) have observed that the rise time of EOG response significantly decreased as the concentration of the odorant solution increased.

Ottoson (1956) described a dose response relationship between the EOG amplitude and the odor stimulus strength of butanol and Coates did similar studies with CO_2. Therefore it is possible that the changing rise time may be due to the influence of the odorant conce4ion and not the characteristic of the responses from olfactory receptor cells.

However in this study the Pattern of rise time did not agree with those of Ottoson (1956) and Coates (1990), though an insignificant difference in decrease of rise time is observed with increased concentration of odorant solution.

Ottoson (1956) also reported that the duration of plateau time and decay time increase as the odor concentration increases. In the present study a change in plateau time is not very significant.

Coates (1990) also reported that the olfactory receptor response to increasing concentration of CO_2 in bullfrogs did not alter the plateau time. According to Coates (1990) these results may be due to the narrow concentration range of odorant solution used in this study, the brief stimulus pulse (0.5s) or may reflect faster elimination of the Odorant from the Epithelium.

The increase in decay time duration with increase in Odorant solution concentration is consistent with the results with Butanol [Gestland (1965) and Coates (1990)]. However the durations of time course (rise, decay plateau time) is different from than those reported with Butanol and CO_2. These differences may be caused by differences in the concentrations of solutions, duration of odorant stimulus and other experimental conditions.

Although in our experiments latency periods varied with concentration of odorants, age of the culture and duration and intervals of stimulus we did not correlate these studies. However some researchers have recorded the latency period which is the time between odor application and responses. According to (Fadool 1992) the latency to response was extremely brief (~ 20ms to 125 ms).These values are much lower to compare with that of latencies reported for dissociated salamander ORNs by Firestein et al (1990) Lack of mucus barrier would hamper stimulus access to the receptor sites. According to Coates (1990) the shorter decay durations of EOG response in CO_2 is due to the removal of CO_2 from the mucosa via enzymes.

In this study the stimulus is being the odorant solution the difference in the decay time must be associated with Ions/Ion channels, transduction feedback mechanism and growth in culture.

Carleton et al (2003) have studied electrophysiological properties including functional maturity and integration into the OB network and kinetics of excitatory an inhibitory synaptic events in newborn olfactory bulb inter neurons. According to them amplitude of spontaneous Inhibitory post synaptic currents remained constant with cell maturation but there was a significant increase in amplitude of sEPSC with maturation.

In contrast EPSC decay times remained similar through maturation and IPSC decay time significantly changed between the granular cells with some dendrites and devoid of spines and the mature granular cells with fully developed dendritic tree and spines. They concluded that IPSC decay times were consistently slower in superficial granular cells (cell body resides closer to mitral cells) compared to deeper GC (neuronal cell body closer to the core of the OB) Thus the two varied time courses of inhibitor events may reflect the differences in the composition of post synaptic GABA release.

As in their counterparts in situ, increasing concentrations of odorant solution evoked increasing amplitude of EOG until to the point of saturation at the highest concentration. A steep slope and a saturation level were exhibited as in a typical dose response curve. A similar test was done in concentrations of Butanol by Gestland (1965) and CO_2 by Coates (1990).

Out of 13 of cultures tested 11 of cultures showed increase in respond amplitude from the lowest concentration and subsequently declined to a saturation point. The peak amplitude of odor evoked response increased with the concentration of the odorant and

saturated at higher levels. Apart from that odorants evoked different amplitudes of responses with the highest for ethyl butyrate in all the cases including acutely obtained rat OE.

When the tissues were subjected to a continuous or high frequency stimulus it was expected to adapt to its environment, as do their counterparts in situ. Adaptation to sustained stimulation appeared to shift the thresholds of the ORNs.

Olfactory system exhibits the same characteristics as adaptation in other sensory systems, like smell and vision. Repeated or prolonged exposure to an odorant typically leads to stimulus-specific decreases in olfactory sensitivity to that odorant, but sensitivity recovers over time in the absence of further exposure. Further, the magnitude of the decrease and the time course of adaptation and recovery are dependent on the concentration of the odor and on the duration of exposure (Pamela Dalton 2000).

The phenomenon of olfactory adaptation has been extensively investigated using electrophysiological techniques. Since research on olfactory adaptation extends to examine cations of cellular and molecular mechanisms it becomes increasingly important to use culture models of common cell lines and also readily and abundantly available. It is also important to relate the findings from these investigations to the characteristics of adaptation obtained from rapidly isolated OE.

For Drosophila larvae that had 5 min of pre exposure to proprionic acid, benzaldehyde or ethyl acetate, the chemo toxic response was significantly suppressed to those odorants relative to larvae that only received pre-exposure to clean air. Importantly, however, these effects appeared dependent upon exposure duration.

The temporal kinetics of recovery from adaptation may differ when viewed at the neural versus the perceptual level. The mechanisms involved in olfactory adaptation have been well explored. Nevertheless, fundamental questions of the adaptation process remain determined. Parallel investigations of adaptation kinetics and mechanisms of adaptation at various levels is now possible in culture models to expand our knowledge about the process of olfactory adaptation - a type of learning that allows organisms to accommodate and to respond to changing environment.

High frequency stimulation with inter pulse intervals of 200 ms, the EOG responses appeared to be a summative effect due to closely spaced inter stimuli intervals. At longer durations of the inter-stimuli intervals (800 ms), adaptation was clearly apparent with the second and succeeding responses of lower amplitude than the initial response. This trend continued and the membrane potential remained depolarized between inter pulse intervals as long as 15 sec However, the adaptation was less pronounced as evidenced by the increased height of the peaks in response to succeeding stimuli.

This trend continued until the duration of the inter-stimuli intervals reached 20 seconds, where the second and succeeding response amplitudes were equal to that of the first EOG amplitude.

Odorant response to brief intervals of stimulus was significantly impaired where as significant recovery of the neurons were observed with sustained intervals between stimuli. Thus we have establish long lived OE cultures models to develop an optical approach for real-time monitoring of odor responses at the sites of primary transduction in olfactory cilia for future research on olfactory mechanisms such as signal transduction,

cell signaling, perception of odorant and how odors are encoded in the brain and many other questions about olfaction which remain controversial and also in biosensors and pharmaceutical studies. With easy access to the system, we would be able to perform real-time imaging on cultures, potentially at repeated intervals, as well as record intra or extra cellularly from receptor neurons and/or bulbar neurons.

4.4 Conclusions

This study shows that long-term culture of OE and OB tissues is possible and promising as a model for the first odorant responsive culture model.

- Odorant responsive olfactory neurons not only survived in culture for 100 days but also yielded typical EOG patterns under prolonged and intensive experimental conditions.
- Factors known to enhance ORN survival may be due to physical contact with olfactory bulb tissue.

The primary conclusion drawn from the results of this study is that the

1. Cultured OE is indeed morphs of their counterparts in situ and their odorant response patterns are of typical of that of acutely obtained OE.

2. The EOG kinetic variation obtained from different age group of cultures may be due to the fact that these culture tissues undergo maturation process in vitro.

3. A dose response EOG shows that cultured mouse OE displays a similar dose response pattern as of dissected rat OE.

4. Responses from cultured OE which were subjected to individual odorants from the odorant mixture of interest matched with their counterparts.

5. Finally this study also shows that these cultured OE are not only comparable to OE in situ but also resilient to survive intense and prolonged experimental conditions like sustained and high frequency odorant stimulations to compare adaptation kinetics of OE of both origins.

Cultured mouse ORNs exhibited typical EOG responses of that of acutely obtained rat and mouse ORNs. A characteristic EOG response consist of a latency, rise, a, plateau and a decay towards the baseline.

The kinetics of EOG varied in different age groups of tissue raised the question of factors involving in rise and decay of EOG responses. In general, the nature of the tissue and the time taken for the removal of odorant from the surface of the OE can play a role in the decay phase of EOG. Since our experimental conditions were same at all times, we could only predict that the nature and some factors involving in the development of OE may play an important role in the rise and decay of the EOG responses.

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5. $G_{\alpha i}$-PROTEIN SUBUNITS INHIBIT OLFACTORY SIGNAL TRANSDUCTION

5.1 Abstract

The odorant-induced accumulation of cAMP can be strongly enhanced by antibodies directed against $G_{\alpha i}$ protein subunits (Sinnarajah et al 1998). Here we report that whole-cell voltage clamp recordings of odorant-stimulated olfactory neurons indicate that endogenous $G_{\alpha i}$ protein negatively regulates odorant-evoked intracellular signaling. Fragments of rapidly isolated rat OE were placed in a recording chamber so that the basal portions were immersed in physiological buffer solution, while the upper epithelial surface with olfactory cilia was exposed to the air. Current responses to air delivered odorant puffs (mixture of ethyl butyrate. eugenol, (+) carvone and (-) carvone) were recorded from individual olfactory receptor neurons with whole cell patch clamp at a holding membrane potential of -67 (±) 5 mV. The patch electrode (resistance of 8 (±3) MΩ) was filled with a buffer solution in the presence or absence of antibodies directed against either the C-terminal sequences of the $G_{\alpha s/olf}$ subunits (Santa Cruz Biotech.), or to antibodies raised against $G_{\alpha i}$-subunits (Calbiochem) or to control antibodies.

Odorant-induced responses were dose-dependent in the range of 0-16 mM and were also dependent on membrane potentials. Respiratory epithelia did not respond to odor stimulation. Antibodies raised against $G_{\alpha i}$-subunits (10– 80 mg/L) caused a strong enhancement of the odorant-induced olfactory responses and showed a remarkable increase in current amplitude with elapsed time. The absolute value of slope of the dose-response curve was steeper at higher antibody concentrations, which may be attributed to an increased rate of perfusion with increasing antibody concentrations. Conversely, intracellular microinjections $G_{\alpha s/olf}$ antibodies inhibited current responses elicited by odorants (20 - 80mg/L) responses obtained showed a remarkable decrease in the amplitude of responses with elapsing time.

The absolute value of the slope of the dose-response curve, however, was steeper at higher antibody concentrations, which may again be attributed to an increased rate of perfusion with increasing antibody concentrations. The net amplitudes of odorant-induced responses were not significantly modified under control conditions (in the presence of intracellular fluid or pre-immune serum). The observed increase in the net amplitude of odorant responses after the blockage of G_i by G_i antibodies further confirms the negative effect Gαi has on AC. When IgG or anti-olfactory marker protein antibody was substituted for G-protein antibodies, the odorant-induced currents were not significantly changed.

The existence of both stimulatory and inhibitory odorant-induced regulation of both generator currents and adenylyl cyclase activity in olfactory cilia may indicate that an initial integration of different odorant stimuli begins at the level of primary reactions

in the same effector enzyme. These data define a novel physiological mechanism for inhibition and/or modulation of olfactory signaling.

5.2 Introduction

Odorant induced olfactory transduction takes place at the cilia of olfactory receptor neurons. Volatile chemicals bind to olfactory receptors, stimulating Gαolf activation of AC, thereby increasing intracellular cAMP levels, which triggers the opening of cyclic-nucleotide gated cation channels (Firestein 1991). An inward transduction current caused by Na+ and Ca++ along with Ca+ induced Cl- current depolarize the cilia. This membrane depolarization can be measured by electrophysiological techniques from ORNs. In the past few decades, morphological, biochemical and classical electrophysiological studies have paved the way to explore and expand the wealth of knowledge about olfaction. Given the complexity of the system itself, many areas of olfaction including olfactory transduction remain a riddle. G protein coupled receptors are a large class of cell-surface receptors that activate specific G protein pathways (Ram P T et al). The best studied G protein pathways are Gi which inhibits adenylyl cyclase, Gs, and Gq, which stimulate adenylyl cyclase and phospholipase C, respectively.

Olfactory signal transduction is initiated when odorants bind to the G protein coupled receptors located in the cilia following which a cascade of events are triggered resulting in

i. The stimulation of adenylyl cyclase (AC)
ii. Formation of cAMP
iii. Influx of cations

iv. And efflux of anions

All of the above events, combined lead to the generation of a graduated receptor potentials and depolarization.

The G-protein bound G_{olf} is centrally involved in the transduction of odorant stimuli, and $G_{\alpha olf}$ deficient mice exhibited dramatically reduced electrophysiological responses to all odors tested (Belluscio et al 1998).Odorant induced G_{olf} cascade is the predominant signal transduction mechanism in olfactory system. The cascade system involves:

(1) Activation of olfactory receptor

(2) Activation of G Protein

(3) Activation of Adenylyl cyclase

(4) Activation of cyclic nucleotide channels and

(5) Accumulation of cyclic nucleotides.

Odorant molecules bind to G protein-bound olfactory receptors (seven transmembrane receptors) present in the olfactory epithelium stimulating the disassociation of $G_{olf\alpha}$ from $\beta\gamma$ (Fung BK 181).

$G_{olf\alpha}$ binds and activates adenylyl cyclase type III (enzyme) which catalyzes the conversion of ATP into cAMP leading to increase in intracellular cAMP levels which triggers the opening of cyclic nucleotide gated cation channels, allowing the influx of cations (Na^{+}, Ca^{++}) which causes membrane depolarization, and the generation of action potentials .

Electrophysiological and biochemical studies indicate that cAMP is the key messenger during olfactory transduction. Dual transduction pathways lead to excitation and suppression of odorant response of OE. Much progress has been made in the studies

of excitatory pathways of this dual transductory mechanism (Jones, L.P 1998). However, the questions regarding inhibitory pathways remain less explored. The heterotrimeric G protein $G_{\alpha olf}$ stimulates adenylyl cyclase whereas; Gαi is responsible for the inhibition of adenylyl cyclase (Sinnarajah et al 1998). Recent studies have made important contributions to our understanding of the increase of cAMP in the cilia in the absence of $G_{\alpha i}$ protein. However, the direct involvement of $G_{\alpha i}$ inhibition on olfactory responses remained obscure. Phosphorylation of receptors; inactivation of G-protein; modulation of adenylyl cyclase activity; and activation of phosphodiesterases;, all of which could result in the decrease in intracellular cAMP concentrations. In addition, the cyclic nucleotide gated channels are subjected to modulation and adaptation.

Therefore a combination of molecular, electrophysiological, biophysical and cell biological techniques are required to delineate odorant transduction.

The objective of this study was to establish the role played by $G\alpha_{olf}$ and $G_{\alpha i}$ protein subunits in the regulation of AC in rat olfactory epithelium. Biochemical studies clearly indicate the inhibitory action of G_i on the cAMP production. This study aims to prove this phenomenon by electrophysiological evidence. Also to be tested in this study was establishment of direct involvement $G_{\alpha i}$ protein on odorant stimulated olfactory responses.

Studies also show variations in electrical activities pertaining to $G_{\alpha olf}$ and $G_{\alpha i}$ proteins in rat ORNs. Most importantly, biochemical studies proved a direct interaction of $G_{\alpha i}$ Protein subunits with ciliary AC.

In addition to these findings, there is a necessity to establish if and how odorant stimulated $G_{\alpha i}$ inhibition of AC takes place in the ORNs. A real time recording of

electrical activity in the ORNs could be a valuable tool to demonstrate the physiology behind $G_{\alpha i}$ mediated inhibition of odorant stimulated olfactory responses. However, there is no electrophysiological evidence to demonstrate these inhibitory phenomena.

5.3 Materials and Methods

5.3.1 Preparation of dissected Rat olfactory epithelium

The intact epithelial preparations were prepared following standard procedures. Rats were anesthetized by injection of pentobarbital and decapitated. The nasal septum was dissected out and olfactory epithelium attached to the septum was trimmed and kept in modified Hanks balanced salt solution (with glucose/without Ca^{++})

5.3.2 Solutions

5.3.2a ECF - (Pseudo extra cellular solution)Hanks balanced salt solution, (modified with sodium bicarbonate and without phenol red, calcium chloride and magnesium sulfate) containing 137 mM NaCl, 5.3 mM KCL, 0.44 mM KH2PO4, 4.2 mM NaHCo3, 3.4 mM NaHPO4, 5.6 mM D.Glucose,pH 7.2). The glass electrode (resistance of 7 – 10 MΩ) was filled with intracellular solution

5.3.2b ICF - (Pseudo intracellular solution): Containing 110 mM KCL, 4 mM NaCl, 2 mM NaHCO3, 1 mM MgCl2, 0.1 mM CaCl2, 2 mM MOPS at pH 7.4.

5.3.2c Odorant solution. (Sigma –Aldrich) A 1600 μM stock solution of the odorant mixture was prepared with deionized water containing 1600μM in 1 L: 0.0016 mols of; - carvone; + Carvone; Eugenol and Ethyl butyrate.

5.3.2d Preimmune serum

A rabbit preimmune IgG was a kind gift of Dr. John Dennis (Auburn University) obtained from Sigma-Aldrich. Specifications: Rabbit serum, Product number R9133, Lot: 041K9089. Slightly hazy, amber liquid containing 0.01% thimerosal. Protein content estimated to be ~ 50mg/ml

5.3.2e Antibodies against $G_{\alpha s/olf}$ - Obtained from Santa Cruz

Specification: Custom ordered L form of antibody that is free of Sodium azide and gelatin. Antibody was dissolved in Phosphate buffered saline (PBS) Gαs/olf (C-18) L. Rabbit polyclonal IgG.

5.3.2f Antibodies against Gαi: Obtained from Calbiochem

Specification: Anti $G_{i\alpha}$-1 and $G_{i\alpha}$-2 Subunits, C-terminal (345-354 and 346-355) (Rabbit) full-line of purified rabbit Polyclonal Antibodies to G-Protein

During experiments, freshly dissected olfactory epithelia were placed in a buffer inside a chamber on a stage with the basal part of the tissue immersed in buffer, whereas the upper epithelial surface with olfactory cilia was exposed to the air. The chamber was connected to the head stage through a recording electrode which closes the circuit during experiments.

Odor stimuli were applied by blowing plumes of volatile odor vapors onto the surface of olfactory epithelium that was exposed to the air. The pressure and duration of the application was controlled by a PV 800 pneumatic Pico pump which uses dried and charcoal filtered nitrogen gas to propel the odor molecules from the container. After the

application of the odor, lingering odor molecules were removed from the vicinity of the tissue by a weak vacuum regulated by the same instrument.

5.3.3 Whole cell recording

Currents were measured with the Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) in the single-cell voltage-clamp mode. Patch pipettes were fabricated using borosilicate capillaries (WPI, Sarasota, FL) and pulled using a P-87 pipette puller (Sutter Instruments, Novato, CA). Pipette resistances were 5- 10 MΩ when filled with the internal solution. Voltage-clamped currents were low-pass filtered at 10 kHz.

Electrical responses propagated by the application of odor were detected and amplified by the amplifier and the data were relayed from the amplifier to the computer through an analog-to-digital interface Digidata 1322A (Axon Instruments). Acquisition, storage and analysis of data were performed using PClamp 9 software (Axon Instruments). Resting membrane potential was measured as the potential at which the current was zero. Experiments were performed at room temperature.

Rat olfactory epithelium was placed in modified Hanks balanced salt solution without Calcium. The tissue was then transferred to chamber filled with the same buffer. A glass recording electrode (7~ 0 mΩ) which was filled with antibodies diluted in Intracellular fluid and secured in an electrode holder, was lowered using a three dimensional micro manipulator, onto the surface of the olfactory epithelium in order to obtain cellular contact.

At this point, the tissue was electrically connected to the amplifier through the ground electrode and the recording electrode which was connected to the head stage by means of a holder established contact with the solution inside the glass recording electrode.

Rat septal olfactory mucosa were dissected out and placed in a perfusion chamber such that the basal portions were immersed in physiological buffer, while the epithelial surface with olfactory cilia was exposed to air.

Patch electrodes of approximately 7 mΩ tip opening were filled with ICF then connected to a patch-clamp amplifier in order to detect responses from the olfactory epithelium.

Once contact between the electrode and the surface of the olfactory epithelium was formed, air puffs of odorant mixture containing 1.6 mM ethyl butyrate, eugenol, and (+) and (–) carvone were applied. Odor responses over the time course of fifteen minutes were recorded after being amplified (Gain=1) by a patch-clamp amplifier and filtered at 2 - 5 kHz. Data were digitized using digidata 1322A (Axon instruments).

Odor was applied through a small Teflon nozzle that has been positioned in the direction of the tissue. A multi-barrel pipette placed downstream from the recording site, was used to deliver odorant stimuli by pressure ejection through a Pico pump. Odorant solution was prepared as 16 mM stock in deionized water to the final volume of 500ml solution.

Electrical responses propagated by the application of odor were detected and amplified by the amplifier instrument. Data are relayed from the amplifier to the

computer through the interface hardware. The responses were then processed and plotted by the computer for real time viewing, as well as for storage and subsequent analysis

5.3.3A Protocol

Voltage clamp recordings were made with the introduction of odorant puffs as stimulus. Each stimulus lasted for 0.5 seconds and the voltage fluctuated between 63 to 70 mV.

By carefully approaching the olfactory sensory neurons with a fine recording glass pipette, it was possible to obtain single-cell voltage-clamp recordings. Recordings were made after stable contact with the olfactory neuron was established.

Stable electrical recordings were obtained from dissected olfactory epithelia. The mean value for the resting potential, V_O, was -70 (±) 3.6 mV and ranged between –70 to -63 mV. Downward responses indicate inward current.

5.4 Results

Whole cell voltage clamp recordings of olfactory neurons were recorded in the presence of $G\alpha_i$ specific antibodies.

The odorant response can be measured in the form of:

(1) EOG, where the response curves represent the receptor potential (field potential) generated across the olfactory ciliary membrane. The measurements were made in mV and recordings were made against elapsed time in minutes.

(2) Whole cell recordings, where the response curves represent membrane current flows across the receptor neuron. The measurements were made in pA and current recordings were made against elapsed time in minutes.

Odorant elicited characteristic membrane depolarization and repolarization phases and the current amplitude depended on the concentration of antibody against time. While the current amplitudes varied from cell to cell, the general features of the response remained constant. As expected, voltage clamped respiratory epithelium did not respond to odorants. With perfusion of olfactory neurons with a patch electrode filled with a solution containing $G_{\alpha i}$ antibodies the odorant evoked currents changed dramatically over time.

Initial current amplitudes did not vary until approximately 5 minutes after perfusion of antibody, after which a progressive increase in the amplitude of the odorant triggered currents were noted.

In contrast, the amplitudes remained almost constant with perfusion of control antibody and ICF. Similar biochemical studies (1) with ciliary preparation and electrophysiological studies with RGS2 antibody administration (2) in olfactory sensory tissues yielded similar results.

Normally, odorants raise intracellular cAMP levels, which opens cyclic nucleotide-gated cation channels resulting in an influx of Ca^{+2} and the opening of Ca^{+2} activated Cl^- channels. The diffusion of $G_{\alpha i}$ antibodies into olfactory neurons should progressively neutralize $G_{\alpha i}$, thereby removing its tampering effect on $G_{olf\alpha}$ stimulated adenylyl cyclase III activity. This leads to elevated cAMP levels, opening of additional cyclic nucleotide gated channels, and increased inward currents as we observed.

Our results were based on the criteria that the excitatory G-protein $G_{\alpha s}$ bind to cell surface receptors leading to the enhancement of adenylyl cyclase and cAMP production

The inhibitory G protein $G\alpha_i$, on the other hand, binds to cell surface receptors causing the inhibition of AC.

5.4.1 Odorant stimulated activation of olfactory responses

Odorant solutions (1 to 16 mM) applied to olfactory epithelium induced a dose dependent response. The response curves were typical with a minimum at 10 mM which increased with increasing concentration to an apparent saturation level of 16 mM (fig 1). Control preparation of charcoal filtered air induced olfactory epithelium did not yield any response. (Figure 1A)

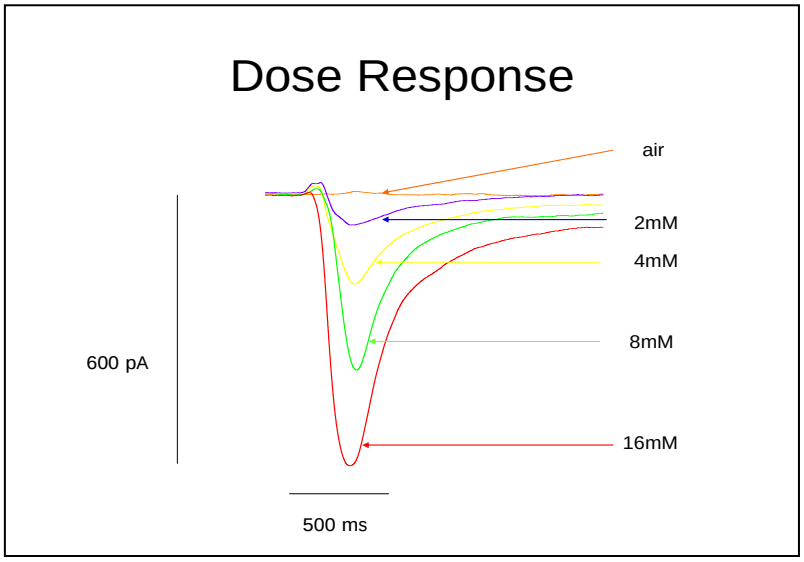

Fig1 A. Current responses to the range of odorant solution concentrations delivered to dissected rat OE. OE was exposed to 1, 2, 4, 8 and 16 mM odorant mixture and dose response showed increased amplitude with increasing odor concentration

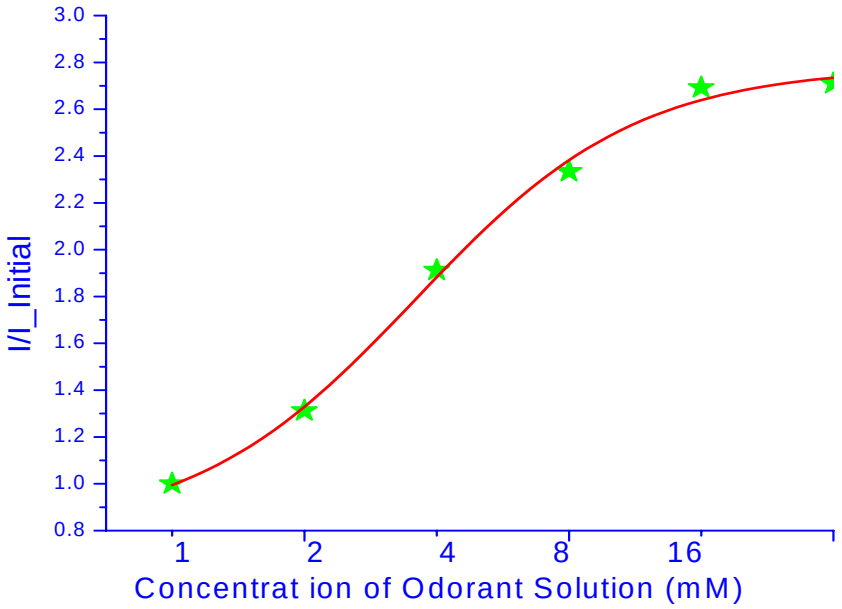

Fig. 1 B) Plot of absolute peak amplitude of the on-responses versus odorant concentration.

Odorant-induced responses were dose-dependent in the range of 0-16 mM. Fig shows plots of peak amplitude of the on-responses versus odor concentration. Peak amplitudes were plotted as a function of the odor concentration

Normalized voltage and current responses: TABLE 1

		EOG	Whole cell
		V/ V_Initial	I/I_Intial
Control	Air	-	-
Concentration of Odorant	1 mM	0.00791	0.24147
	2 mM	0.18421	0.51678
	4 mM	0.50471	0.85603
Solution	8 mM	0.83463	0.99819
	16 mM	1	1

5.4.2 Odorant induced enhancement and inhibition of olfactory responses.

Antibodies directed against $G_{\alpha s/olf}$ inhibited olfactory responses stimulated by odorant solutions in a dose response manner (Figure 2).

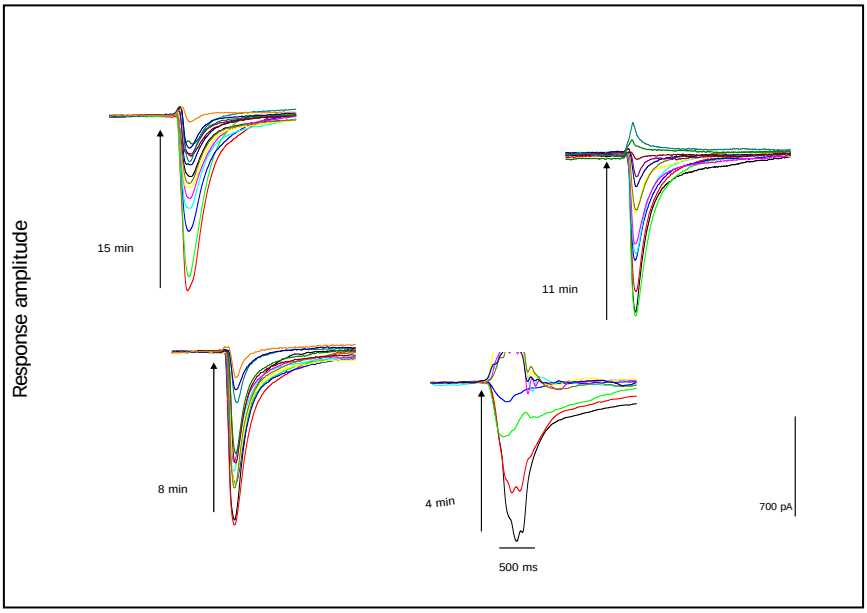

Fig 2 Odorant Induced current traces with glass electrode containing Gαolf Antibody diluted with ICF (2 g/L to 8g/L) from whole cell voltage clamp recordings of olfactory neurons with a holding potential ~ -70 mV. The stimulus was a 0.5 sec Pulse of odorant mixture

Increasing concentrations of $G_{\alpha s/olf}$ antibody treatment with time, yielded sharp decrease in odorant responses and showed a steeper slope towards the baseline at higher antibody concentrations. In contrast, antibodies against $G_{\alpha i}$ subunits caused strong enhancement of odorant induced olfactory responses in a dose dependent manner. Increasing

concentrations of $G_{\alpha i}$ antibody with time, yielded sharp increases in odorant responses with steeper slopes at higher antibody concentrations.

5.4.3 Odorant induced Currents with Gαi Antibody Induction

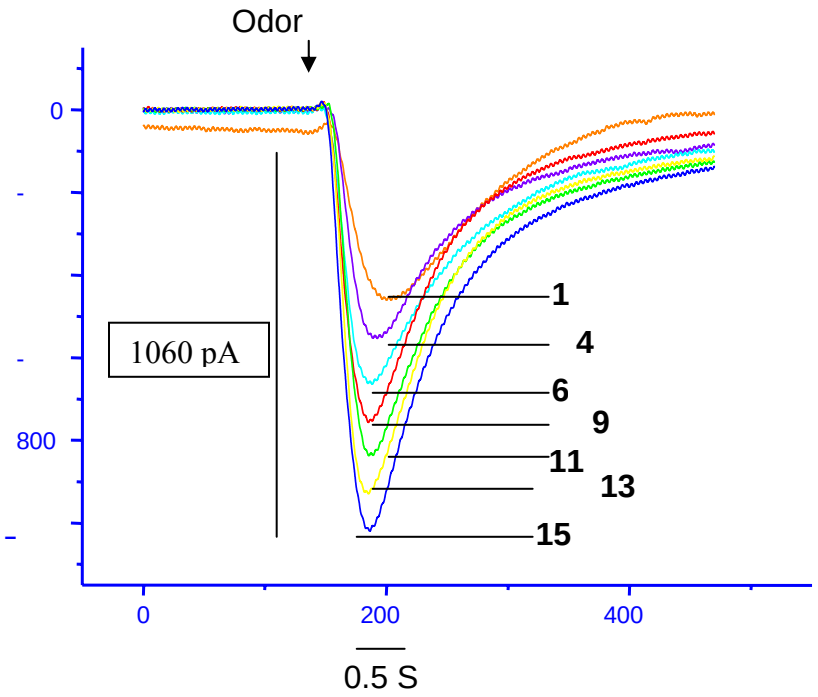

Figure 3 shows odorant induced currents recorded with patch electrodes filled with G_{olf} antibodies (4 mg/L). Odorant puffs were delivered at 1, 4, 7, 13 and 15 min after initiating the perfusion as indicated. The cell was clamped at -70 (±) 3 mV. Odorant puffs were delivered at 1, 6, 9, 12, and 15 minutes after initiating the G_i antibody perfusion as indicated.

Perfusion of Gαi Antibody to Rat olfactory neuron

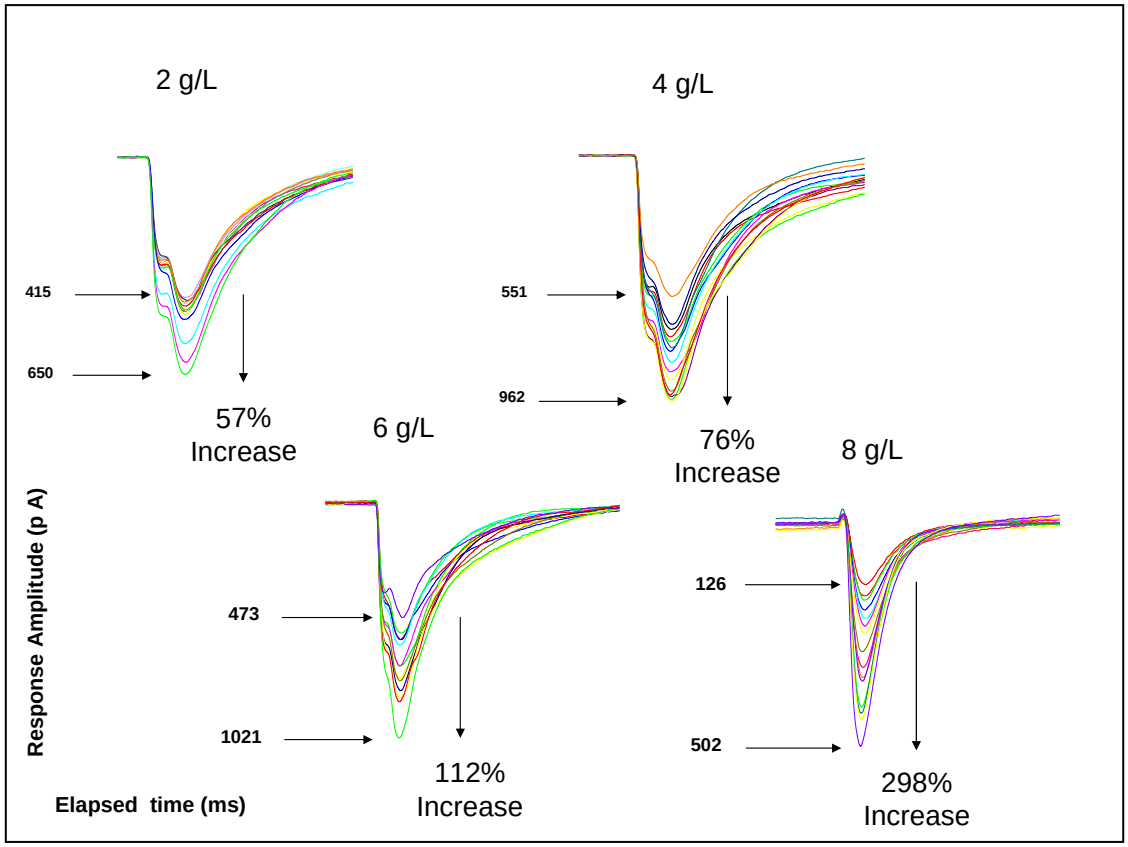

Figure 4 shows the whole cell voltage clamp recordings of olfactory neurons obtained with antibodies against Gαi. Attenuation of odorant signaling was observed as can be seen from the figure.

Odorant induced current traces with glass electrode containing $G_{\alpha i}$ antibody diluted with ICF (2 mg/L to 8mg/L) from whole cell voltage clamp recordings of olfactory neurons with a holding potential ~ -70 mV. The stimulus was a 0.5 sec pulse of odorant mixture. Downward direction indicates inward current.

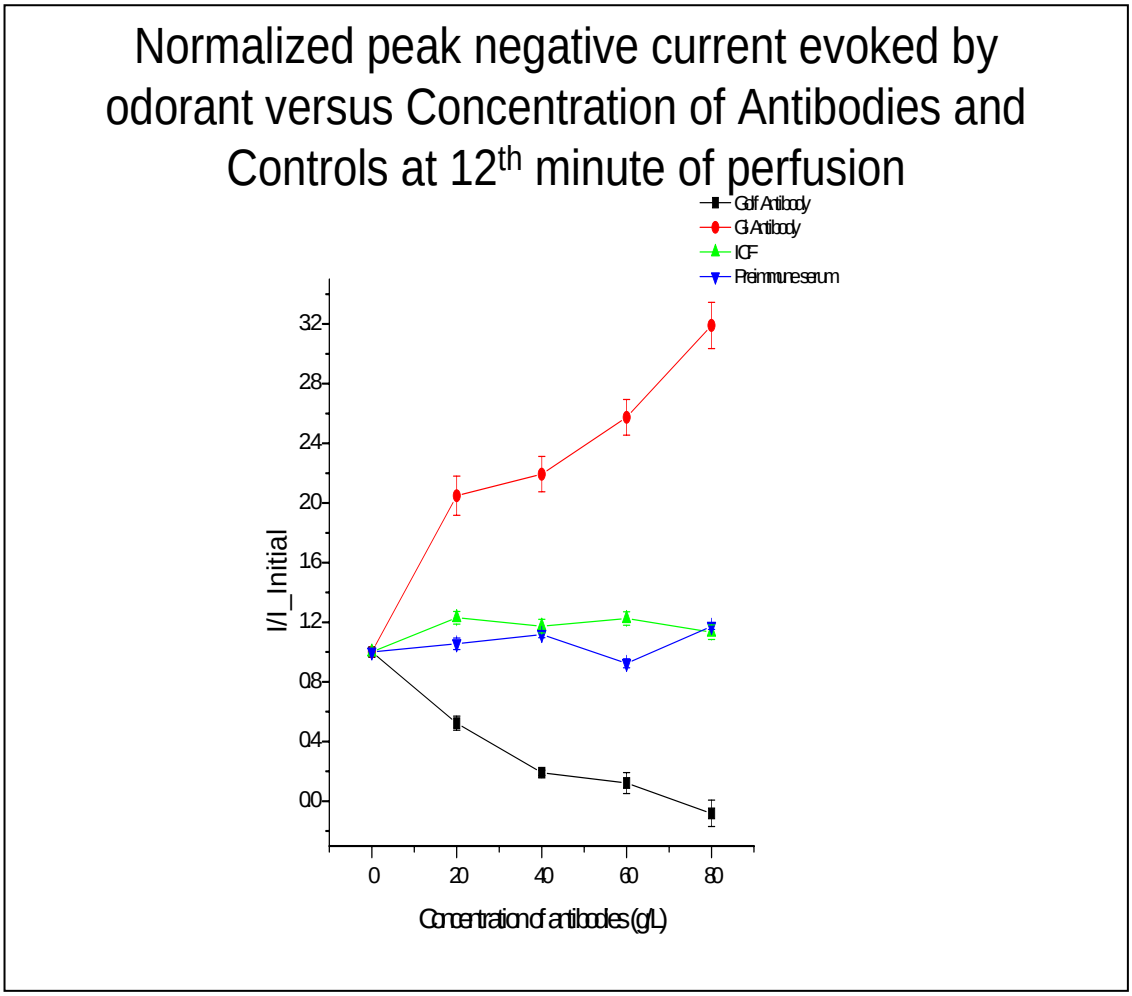

Fig. 5 Plot of normalized peak negative currents (I/I_initial) versus concentration of solutions of interest at 12th minute of perfusion. The current V_s concentration of solution of the peak currents of odor responses plotted as a function peak amplitudes.

Normalized peak negative current evoked by odorant versus perfusion time with Antibodies against $G_{\alpha i,}$ $G_{\alpha olf}$, Preimmune serum and ICF

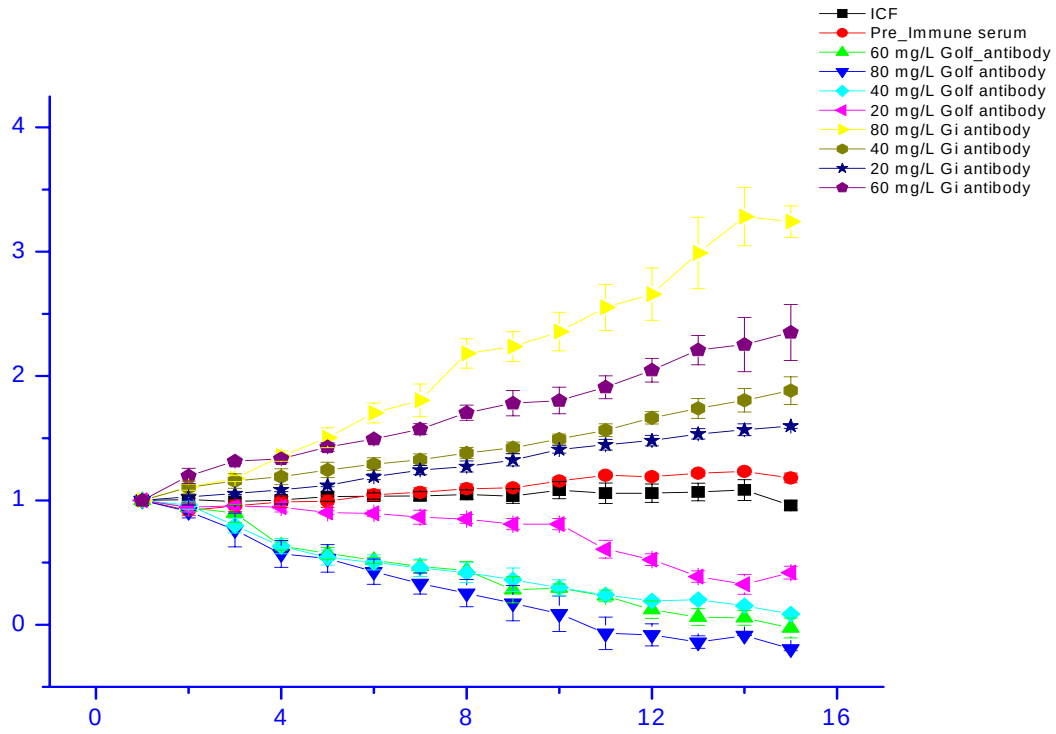

Figure 5b shows the plot of normalized peak negative current ($I_{peak}/I_{initial}$) evoked by odorant versus perfusion time. Normalized peak currents of control experiments after perfusion with: Gi antibody, $I_{initial}$ = –450.2 pA; buffer, $I_{initial}$ = –375 pA. Preimmune serum, $I_{initial}$ = –94.7 pA; and pre-immune serum, $I_{initial}$ = – 520 pA are also shown.

The effect on odorant induced $G_{olf\alpha}$ signaling by antibodies against $G_{olf\alpha}$ and $G_{\alpha i}$ was measured in rat OE. In the presence of antibodies against $G_{\alpha i}$, odorants triggered a 4-fold increase in olfactory responses, while similarly adding $G_{olf\alpha}$ substantially decreased odorant induced olfactory responses. At 40 mg/L antibody concentration against $G_{olf\alpha}$ reduced olfactory responses to 50% from the initial response (fig 6).

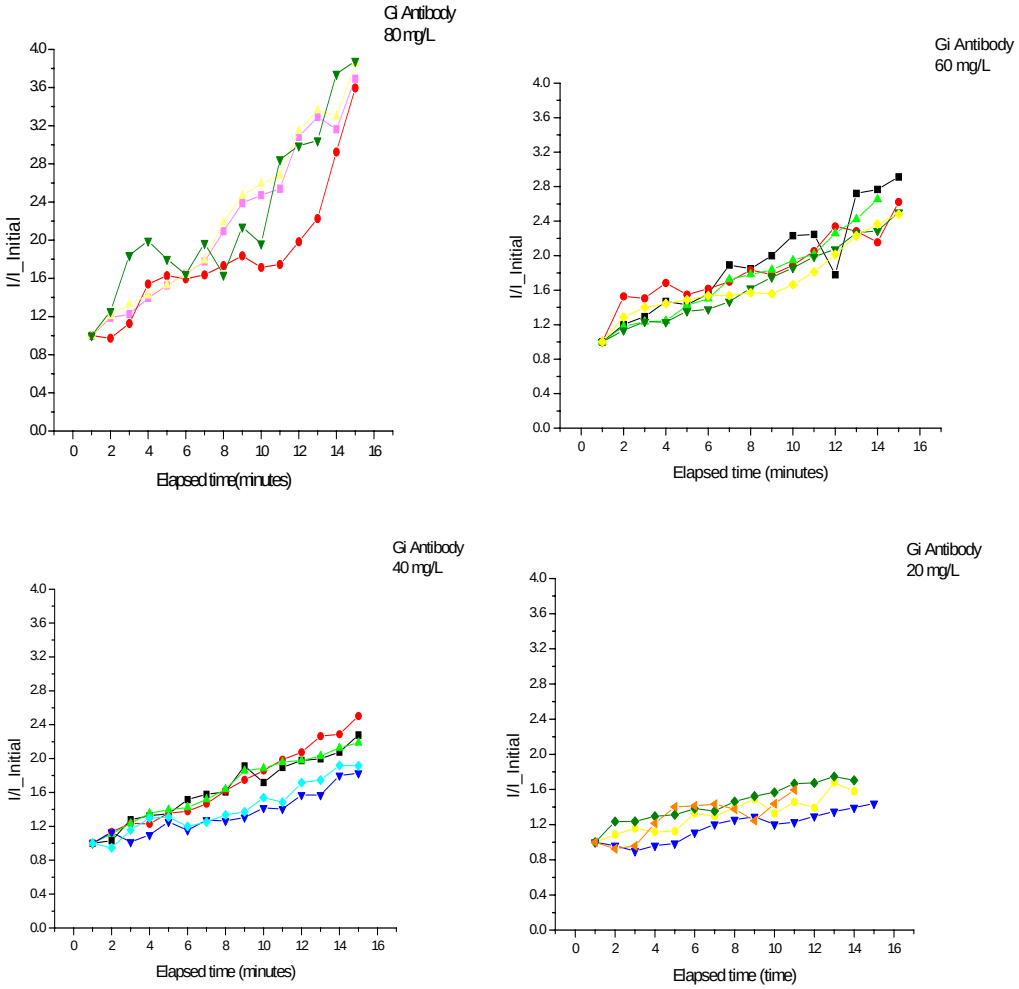

Fig 6 A Plot of normalized current amplitudes (I/I_ Initial) evoked by odorants versus perfusion time with Gαi antibody mixture. (2g/L to 8 g/L).

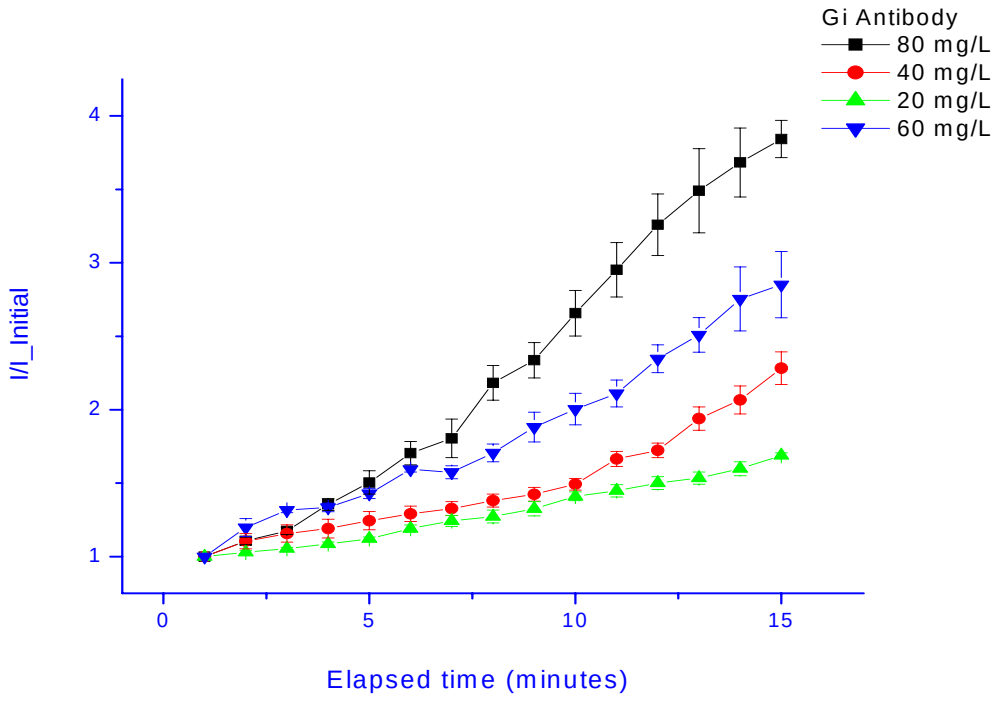

Fig 6 B Shows average normalized peak currents evoked by odorant versus perfusion time with **(A)** antibodies for $G_{\alpha i}$ subunits with increasing concentrations.

At 7^{th} minute after perfusion of $G_{\alpha i}$ antibody (40mg/L), the odorant induced olfactory responses were more than twice as high compared to odorant responses stimulated by the control, ICF (figure 6). When preimmune serum diluted in buffer solution was substituted for antibodies, there was no significant changes in the odorant induced olfactory responses (fig 6). Perfusion of ICF alone in the neurons yielded constant amplitude of odorant response with time (fig 6B).

$G\alpha_i$ reduces cAMP production during odorant-stimulation by acting as an inhibitor of adenylyl cyclase type III, the predominant adenylyl cyclase isoform in olfactory neurons (6). Whole cell voltage clamp recordings of odorant stimulated olfactory neurons indicated that endogenous $G_{\alpha i}$ negatively regulates odorant-evoked intracellular signaling.

The enhanced odorant response by suppression of $G_{\alpha i}$ protein seems to be realized by increased cAMP concentration. Although previous biochemical studies have demonstrated a $G_{\alpha i}$ controlled inhibition of odorant induced cAMP synthesis, it was not proved electro-physiologically with odorant induced response of olfactory neuron.
These electrophysiological findings were consistent and correspond well to the western blotting and immunoelectron microscopy results obtained from homogenized ciliary preparation of rat olfactory epithelium and cilia-enriched preparations from frog and rat olfactory neuroepithelia.

These results reveal a new mechanism for controlling the activities of adenylyl cyclase, which likely contributes to the exquisite ability of olfactory neurons in discrimination or modulation of odorants.

The constitutive expression of $G_{\alpha i}$ in olfactory neurons along with the previous biochemical data suggested that the endogenous levels of $G_{\alpha i}$ could modulate odorant responses.

5.5. Discussion

The levels of intracellular cAMP production are regulated through the enzyme AC which converts ATP to cAMP. AC is directly activated or inhibited by G_{α}-proteins and some isoforms of AC are regulated by Ca^{++} and calmodulin. Activation of G_s coupled GPCR in turn, activates $G_{\alpha s}$ molecules which has a positive effect on AC resulting in the catalysis and the conversion of GTP into cAMP. The direct activation of a cation permeable channel by cAMP is the final step in producing the odor induced ionic current. In the presence of normal physiological extra cellular Ca^{++}, the second messengers (cAMP) elicits the opening of the channel allowing Ca^{++} to flow in, and this increase in intracellular Ca^{++} concentration appears to activate a chloride current that helps depolarize the olfactory cell.

Thus, the cyclic nucleotide gated channels plus the Cl^- evokes whole cell current that results in the signal transduction (R.Taussig 1994). The cAMP then binds to intracellular protein kinases, initiating phosphorylation events that regulate target enzymes. On the other hand, $G_{\alpha i}$-coupled receptor activation activates $G_{\alpha i}$; a molecule which exerts an inhibitory effect on AC.

It is understood that the cAMP PDE enzymes act as an important negative-feedback system on the signal transduction cascade, in order to regulate intracellular cAMP concentrations. (Wiechen, K 1995). Adenylyl cyclase can be both stimulated and inhibited by G proteins (Sinnarajah 1998).Although, direct inhibition by Gαi subunits has always proven difficult to demonstrate.

Few studies have shown direct inhibition by $G_{\alpha i}$ - subunits (Rau T 2003 Janssen PM 2002). Antibodies specific to $G_{\alpha i}$ have been able to suppress receptor mediated inhibition of adenylyl cyclase (Rau T 2003). Studies (P G Feinstein 1991) on cloned cDNAs encoding six distinct mammalian adenylyl cyclase demonstrated inhibition of adenylyl cyclase by all three Gαi subunits.

Organisms can detect odorants at micro- to nano and pico molar range. When electrophysiological studies are conducted using micro- to milli molar levels of stimuli (as was the case in this study); it is always a question if the concentration of odorant are being in the "physiological range." However, the actual concentration at which the odorant molecules reach the immediate vicinity of an OR in a living organism varies. Olfactory cells in situ are surrounded by mucus that has substances such as odorant binding proteins that may assist in the delivery (or removal) of odorant molecules to the ORs. When experiments are conducted in vitro, the normal external components of the cell are altered. Thus, the equivalent stimulus intensity in vitro to the nano and pico molar levels reported in vivo is not known.

Also electro physiologists often use micro molar stimulus concentrations for in vitro experiments to obtain a reasonable number of observable cell responses, whether this is due to the viability of olfactory cells *in vitro* or due to the lack of the appropriate extra cellular milieu remains to be determined.

In situ, olfactory neurons must be able to function over a wide range of stimulus intensities and cope with the effects of strong odorant stimulation. The olfactory receptors are one of the largest groups of G-protein coupled receptors described to date.

In this study, in order to obtain optimum results for odorant induced electrical activity, a mixture of ethyl butyrate, - carvone, + carvone and eugenol were used as odorant solutions.

This mixture has proven to elicit significant responses in previous electrophysiological studies (Sinnarajah et al 2001, Josephson et al 2004). The odorant solution of interest produced increasing amplitude of current and voltage responses with increasing concentration of odorants in a dose dependent manner. Signal amplitudes were significant at 2 mM and reached an apparent saturation at an odorant solution concentration of about 16 mM. A dose dependent study on ciliary preparation (Sinnarajah 1998) showed that the cAMP production were dependent upon the presence of GTP, indicating that cAMP were mediated by G proteins.

Antibodies raised against $G_{\alpha i}$ strongly enhanced odorant induced olfactory responses, whereas antibodies raised against $G_{\alpha olf}$ resulted in inhibition of olfactory responses. These results are consistent with a study on rat olfactory ciliary preparation (Sinnarajah 1998) where antibodies directed against $G_{\alpha i}$ subunits strongly enhanced AC activity while antibodies against $G_{\alpha olf}$ on the other hand, inhibited AC activity. Studies on the presence of AC III isoform in the olfactory epithelium (Bakalyar et al 1990) which is thought to be activated by G_{olf} (1) and AC activation by exogenous $G_{\alpha s}$ in rat OE (Pfeuffer 1989) also supports these results.

Sinnarajah et al (1998) reported evidence that a single effector enzyme, adenylyl cyclase, in cilia from rat olfactory epithelium, can be coupled with both activator and inhibitor G-proteins. However, physiological studies on olfactory receptor

neurons demonstrate that when stimulated with odorants, olfactory neurons can generate excitatory or inhibitory responses, or a combination of both.

The present study supports the nature of odorant-elicited inhibition and suppression and also shows that odor induced olfactory cell responses can be excitatory, inhibitory, and suppressed by the application of odorants at relatively high concentrations. Suppression may occur in some cells and affects the net current that is generated by the cells in response to odorant stimuli.

Furthermore, some cells respond to odorant mixtures with all three types of responses: excitation, inhibition, and suppression although it is not proven whether individual odorants can produce all three effects or whether each component of the mixture produces one type of response and the net output of the cell integrates these individual response types. If odorants act through $G_{\alpha i}$ proteins to inhibit adenylyl cyclase during initial stages of the odorant-mediated cascade in olfactory cilia, then one must be able to establish their physiological role. These criteria are similar to those suggested for justifying claims that a given effector produces its effect as a result of the stimulation of adenylyl cyclase (Robinson et al 1971). The main criteria are as follows:

(i) The olfactory receptor neurons should contain $G_{\alpha i}$ proteins,

(ii) The activity of the type III adenylyl cyclase (which is the predominant form in olfactory cilia) can be modulated by $G_{\alpha i}$ proteins, and

(iii) The adenylyl cyclase inhibition should not occur in response to odorants when $G_{\alpha i}$ proteins are blocked or inhibited. Criterion (i) is supported by immunohistochemical observations in rat and other species.

The presence of G_i-proteins has been reported in the lobster olfactory organ (Hatt H 1994), in chemosensory membranes from the channel catfish (Hansen et al 2003), and in the nasal epithelium of the frog (Pace et al 1986) and rat (Cortés et al 1988). Although all three Gαi-subunits (Gαi1, Gαi2, Gαi3) were present in rat olfactory neuroepithelium, only Gαi2-subunits were so far reported in the rat olfactory cilia [Jones 1989]. Western blot analysis and immuno-electron microscopy confirms the presence of $G_{\alpha i}$-subunits in our olfactory cilia preparations (Fig. 3). Evidence in support of criterion (ii) is provided by a study of muscarinic m4 acetylcholine receptors and type III adenylyl cyclase co-expressed in human embryonic kidney cells (Tso 2001). In that study, it was shown that the adenylyl cyclase III was inhibited due to receptor coupling through Gαi. Our results are also consistent with criterion (iii). When $G_{\alpha i}$-subunits were bound by specific antibodies we observed an apparent block to $G_{\alpha i}$ function, which produced an increase in cAMP content, indicating that adenylyl cyclase inhibition was reduced (Fig. 2A).

The dual regulation of the adenylyl cyclase activity we observed in our experiments was different from that with two different effector enzymes, adenylyl cyclase and phospholipase C (Fadool et al 1992).

We have presented evidence that a single effector enzyme, adenylyl cyclase, coupled with activating and inhibitory G-proteins controls the level of a single second messenger, cAMP.

This bi-directional regulation of certain adenylyl cyclase is explained by the presence of independent sites for interaction with stimulatory $G_{\alpha s}$ and inhibitory $G_{\alpha i}$ subunits (Tausing et al 1994). $G_{\alpha i}$-subunits are present in olfactory cilia. The adenylyl cyclase enhancement in the presence of $G_{\alpha i}$-antibody is controlled by odorants and GTP, and cannot be controlled by βγ-subunits (Tang et al 1991), $Ca2^{+}$-calmodulin, or phosphodiesterases. We therefore hypothesize that adenylyl cyclase of olfactory neuron cilia is inhibited functionally through a coupling with a $G_{\alpha i}$ protein pathway. This mechanism of inhibition may be important for understanding the attenuation of odorant-stimulated cAMP levels in olfactory neurons.

Through biochemical studies, it has been established that:

(a) Odorants act through $G_{\alpha i}$ proteins to inhibit AC during initial stages of the odorant mediated cascade in olfactory cilia.

(b) $G_{\alpha i}$ Proteins are present in olfactory neurons.

(c) The activity of AC 111 can be modulated by $G_{\alpha i}$ Proteins and

(d) The adenylyl cyclase inhibition takes place in response to odorants when $G_{\alpha i}$ proteins are inhibited. If odorant induced adenylyl cyclase inhibition did not take

place when $G_{\alpha i}$ proteins are inhibited, then one must be able to demonstrate certain criteria to establish a direct effect it has on olfactory responses. The criteria are:

i) The odorant induced olfactory responses are enhanced in a dose dependent manner by both odorant mixture and antibodies against $G_{\alpha i}$ subunits

(ii) Odorant induced inhibitory olfactory responses by antibodies raised against $G_{\alpha olf}$ showed dose dependency.

(iii) Controls ICF and preimmune are not enhanced in a dose dependent manner to odorant stimulation.

It has been also demonstrated that GTP enhanced AC in a dose dependent manner in olfactory cilia (Bruch et al 1987). A similar study by (Sinnarajah et al 1998) also showed GTP enhanced dose dependency of AC in rat olfactory ciliary preparation up to a saturation level of 10μM of GTP, beyond which the level cAMP concentration declined. It is speculated that high concentration of GTP may result in the modulation of cAMP production through activation of G_i at higher GTP concentrations. A preliminary study (Sinnarajah et al 1998) indicates the possible activation of G_i at high GTP levels, which may in turn take part in the down regulation of AC. The presence of $G_{\alpha i}$ subunits in the olfactory ciliary preparations has been established through immuno-electron microscopy, and western blot studies (Sinnarajah et al 1998).

The presence of $G_{\alpha i}$ subunits in OE is also confirmed by Jones and Reed, (1987) Modulation of AC111 by $G_{\alpha i}$ proteins is supported by a study in human embryonic kidney cells (de Almeida 1994**),** co-expressed with muscarinic m4 acetyl choline

receptors and type 111 AC. The study showed inhibition of AC111 through receptor coupled $G_{\alpha i}$. Another study (de Almeida 1994) also supports inhibition of cloned AC111 by $G_{\alpha i2}$ subunits in a transfected system. Immunohistochemical studies (Sinnarajah 1998) clearly showed that specific antibodies against $G_{\alpha i}$ subunits interrupted the inhibitory effect of G_i proteins on AC. Thus it is evidently established that a single effector enzyme, AC can be coupled with inhibitory and excitatory G proteins and to control the level of intracellular cAMP concentration.

This dual regulation of some AC isomers is explained by the existence of independent sites for interaction with stimulatory and inhibitory G protein subunits. (Fadool et al 1992). This study establishes the inhibitory and excitatory effect of AC mediated dual transduction system with support of previous studies especially with ciliary preparation of rat OE (Sinnarajah 1998). In olfactory signal cascade, ciliary AC plays a vital role along with odorant receptors, G proteins and CNG channels.

The integrity of these vital elements during olfactory transduction has been demonstrated by a functional reconstitution of olfactory ciliary membranes activated by odorants or cAMP. The reconstituted membranes displayed typical single channels fluctuations exhibited by native olfactory neurons (Firestein et al 1994). Odorant induced activation of reconstituted ciliary membrane required the functional existence of components of the olfactory cascade including odorant receptors, G Proteins, AC, and CNG channels (Vodyanoy et al 1991).

Thus a similar dual transduction system may be functional in olfactory cilia insitu. A combination of biochemical and electrophysiological studies on the dual

regulation of AC on inhibition and enhancement of olfactory responses may be of importance in olfactory modulation a phenomena, necessary for odor perception in a changing environment. In conclusion, odorant stimulated AC through excitatory G protein (G_{olf}) results in enhancement of olfactory responses.

On the other hand odorant stimulated inhibition of AC takes place through inhibitory G protein (G_i) which results in the modulation of cAMP production. The enhancement of AC by $G_{\alpha i}$ antibody can be controlled by odorants and GTP but not by $\beta\gamma$- subunits (38), ca^{++} - calmodulin or phosphodiesterase.

Also immunohistochemical studies (Sinnarajah et al 1998) demonstrated dose dependency of $G_{\alpha i}$ and G_{ol}f against cAMP production which clearly stated the direct link between AC and the G_i, G_{olf} Proteins. The phenomena of inhibition and enhancement of odorant stimulated responses indicates the integration and discrimination of odor stimulus begins at a very early stage of olfactory perception. This mechanism of inhibition may explain the modulation of odorant stimulation a phenomena, necessary for odor perception in a changing environment.

Thus an electrophysiological study showing a direct involvement of AC inhibition by $G_{\alpha i}$ in odorant responses was demonstrated for the first time in this study.

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6. COMPOSITIONS AND METHODS FOR ENHANCEMENT AND STABILIZATION OF OLFACTORY RESPONSE TO ODORANTS

6.1 Abstract

The electrophysiological effects of using certain metals on OE were tested with EOG and whole cell techniques. Commercial colloidal metal suspensions such as zinc (Zn), copper (Cu,) gold (Au) and silver (Ag) and a biological product derived from shark's blood, proteon neucleating centers (PNC) with high Zn content were individually mixed with odorant solution of interest to be tested. OE was subjected to plumes of odorant mixtures with metals of interest as an ingredient and electrophysiological measurements were made in the form of EOG. In addition whole cell experiments were also conducted during which the metals suspensions were filled in the glass electrode and were directly injected intracellulary into the OSN. Voltage clamped current recordings were made over a time period to observe the change in amplitude with perfusion of metals. Control studies included exposure of air to the OE and injection of ECF alone through the glass electrode during EOG experiments. When olfactory epithelium was subjected to PNC and Zn the EOG amplitude showed dramatic increase over a 15 minute recording period. There was no EOG activity at all when the OE was subjected to plumes of air alone with ECF in the electrode.

The results obtained in the whole cell configuration were consistent with the EOG results and in both conditions PNC and Zn suspensions enhanced odorant stimulated olfactory responses.

Conversely, exposure of Cu, on OE did not significantly modify the net EOG amplitudes with elapsing time. Exposure of Au and Ag appeared to damage the OE. The EOG's obtained with Au and Ag were irregular and short lived. The observed increase in the net amplitude of odorant responses with odorant puffs containing PNC and Zn to the olfactory neurons confirms the positive effect of PNC and Zn on Olfactory responses. These data define a novel physiological mechanism for enhancing olfactory sensing/responses. A detailed understanding of the effect of these crystalline metallic nanoparticles (PNC) will be vital for creating a practical olfactory enhancement model.

6.2 Introduction

The use of Zn has been prevalent from early times. Medicinal skin cream with Zn as an ingredient was cited in Egyptian papyri from 2000 BC, and zinc has evidently been used throughout Roman and modern times. The lotion "Calamine" was named for one of its ingredients, zinc ore. (Frederickson et al 2005).

Apart from this appreciation of its biological importance, it is becoming increasingly evident that Zn functions as a signal ion in a number of physiological functions and acts as pharmacological target, which makes Zn a candidate of the research object in the field of cell signaling for years to come.

Zn deficiency, either dietary (Alpers 1994), or caused by treatment with histidine (Henkin et al. 1975), thiocarbamides (Erikssen et al 1975) or captopril (Zumkley et al.

1985) was unique in causing a complete and rapidly reversible anosmia. Zn plays a significant role in neuronal functions such as synaptic discharge of Zn from certain neurons for example, cerebral glutamatergic neurons.

Free Zn^{++} in biological media is toxic and their concentration in tissues and the regulatory mechanisms are yet to be defined. During brain injury (stroke), neurological disorders like Alzheimer's disease, intracellular free Zn^{++} increases up to several times in the neurons from normal levels. Pharmacological treatment to buffer the free Zn ions can prevent the death of the affected neurons. Investigated clinical models show some encouraging signs that the method of treatment was effective. Zn is involved in cell signaling and is secreted by many other cell types other than brain. (Frederickson et al 2005).

6.2a Zinc in CNS

Neurodegenerative disorders like Alzheimer's disease, Parkinsons disease, familial amyotrophic lateral sclerosis, and the transmissible spongiform encephalopathy, show common pathological conditions of misfolding and aggregation of specific proteins. (Stojanovic et al 2004).

6.2b Zinc in peripheral nervous system

6.2b (i) Vision

Zn^{2+} deficiency is also known to cause retinal neurodegeneration and night blindness. Lack of physiological levels of Zn due to mutation of Zn binding sites of the rhodopisn, the G protein coupled receptor causes misfolding and withering, leading to cell death and degeneration of retina and sometimes resulting in blindness. The critical importance and the physiological relevance of the Zn^{2+} coordination site (within the

rhodopsin trnasmembrane domain) to the function of rhodopsin have been reported (Ugarte et al 2001, Stojanovic et al 2004). The same studies also confirm that Zn^{2+} is bound to rhodopsin and that the coordination site has a high-affinity to Zn; and that a Zn^{2+} coordination site in the transmembrane domain is selective for Zn^{2+} and plays a critical physiological role in rhodopsin stabilization in the dark.

Also they suggest that, in dark, the Zn^{2+} coordination sites in the rhodopsin transmembrane domain performs a stabilizing mechanism, retaining rhodopsin in the inactive conformation. Upon photo activation, this Zn^{2+} coordination is disrupted, allowing the unhindered formation of photo activated intermediates.

6.2b (ii) Olfaction

The ORs are seven-helix transmembrane proteins, with an odorant-binding site on the cell surface and a G protein-binding site in the cytoplasm. Studies are underway to establish a definite structure of the ORs. Wang et al have established both computer modeling and experimental evidence for the binding of metal ions to a consensus sequence found in ORs.

Wang et al (2003) have also hypothesized that metal ions play an important role in odorant recognition and proposed a metal-binding site in the loop between fourth and fifth helix. According to Wang et al , a synthetic pentapeptide that contains this putative metal binding site has high affinity for binding Cu^{++} and Zn^{++} ions, and also undergoes a striking transition to an α-helical structure upon metal ion binding. They proposed a "shuttlecock" mechanism for the potential structural change in ORs upon odorant binding.

Human olfactory system can detect ligands for metal ions (amines), methylamine at 18 ppb and (thioles) methylthiol at less than 1 ppb but can detect alcohol (Devos et al 1990) at only about 100 ppm. These differences in odorant affinity difference can be best explained by coordination to metal ions like Cu^{++} and Zn^{++} bound in an OR. Cu and Zn have strong amine and thiol-binding property and are frequently found in metalloproteins. (Wang et al 2003).

6.2c Electron tunneling

Luca Turin (1996) proposed a process called "inelastic electron tunneling" to explain the Zn involved odorant perception. Electron tunneling is the transfer of electrons down the backbone of the protein. Certain classes of odorants bind to Zn readily. Indoles bind to Zn and are very strong odorants and if the Zn ion at or near the electron tunneling site will increase their effective concentration at the receptor. Apart from the active role of Zn binding in transduction process, metal binding could be used to enhance fragrance if it were possible to build into the molecule to increase odorant strength. (Turin 1996). Intra-nasal Zn gluconate gel is marketed as a remedy for common cold. However, intranasal Zn (the exposure of olfactory epithelium to Zn cation) has been reported as a cause of anosmia in humans and animals. (Alexander et al 2006).

In summary, Zn is an essential trace element in numerous neurophysiologic functions. Thus, both Zn deficiencies and over expression can be lethal to biological tissues. With the advancement of scientific techniques in addition to the growing interest in Zn as a component in cell signaling, it's no doubt that this decade will answer many questions in cell signaling which remain a riddle.

6.3 Materials and Methods

Chemicals: All chemicals were obtained from Sigma –Aldrich.

6.3a Odorant solution

A 1600 μM stock solution of the odorant mixture was prepared with de-ionized water.

6.3b ECF - Physiological buffer : Modified Hanks balanced salt solution, (With sodium bicarbonate. without phenol red, calcium chloride and magnesium sulfate - and containing 137 mM Nacl, 5.3 mM KCL, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 3.4 mM $NaHPO_4$, 5.6 mM D.Glucose, pH (7.2). The glass electrode (resistance of 7 – 10 MΩ) was filled with intracellular solution

6.3c ICF- (Pseudo intracellular solution)

Containing 11 0mM KCL, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM MOPS at pH 7.4.

6.3d PNC (Proteon nucleating centers):

PNC was obtained from shark blood diluted with purified water and obtained with subsequent preparations.

6.3e Zinc, Copper, Gold and Silver: (Inspired Technologies)

Electro processed and chemical free colloidal Zinc (plus) and premium grade copper (8fl.oz/240ml) was used in the studies.

6.3f Preparation of Dissected Rat Olfactory Epithelium

The intact epithelial preparations were prepared following standard procedures. Rats were anesthetized by injection of pentobarbital and decapitated. The nasal septum was dissected out and olfactory epithelium attached to the septum was trimmed and kept in modified Hanks balanced salt solution (with glucose/without Ca^{++})

Longevity: Longevity of the tissue under experimental conditions varied with individual animal and usually in the range between 4 to 6 hours.

6.3g Electrodes

Glass patches electrodes (World Precision Instruments, Inc., Sarasota, FL) of approximately -24 μm tip size for EOG and -1μm for single cell recording were used in the studies.

6.4 Method in brief

To establish the acute effects of Zn metal nanoparticles on OE, whole cell and EOG recordings were made with or without Zn. Rat olfactory epithelia were dissected and prepared for EOG and single cell recording using standard techniques.

Once the olfactory tissues were properly placed in the recording chamber (with the epithelial surface exposed to the air), odorant vapors as well as odorant /metal nanoparticle mixtures were delivered to the odor receptors. Olfactory stimulation of rat olfactory epithelium was measured using Electro-Olfactography (EOG) and single cell current recordings.

Another embodiment of this study was the method for incorporation of the metal nanoparticles with the odorants of interest and delivery of the odorant /metal nanoparticles mixtures to the odor receptors.

It was unexpectedly found that the odorant/metal nanoparticles mixture could be delivered out of an aqueous media and result in significant stimulation of the olfactory neurons as evidenced by EOG and single cell responses. The resulting responses were recorded and subsequently analyzed.

6.4a EOG experiments

Rat or dog olfactory epithelia dissected out immediately after decapitation were placed in Hank's balanced salt solution (with glucose). The tissue was then transferred to a temperature regulated chamber filled with the same buffer using a 3-dimensional micromanipulator. A glass patch-electrode (0.5 MΩ resistance), which was filled with Hank's balanced salt solution for control experiments and subsequently with PNC, Zn, Cu, Au and Ag and secured in an electrode holder, was lowered onto the surface of the olfactory epithelium until it made contact with the surface.

At this point, the tissue was electrically connected to the amplifier through the ground electrode and the recording electrode (inside the glass patch-electrode). Odor was applied through a small teflon nozzle that had been aimed in the direction of the tissue. Dose dependency EOG measurements were obtained by the incorporation of the metal nanoparticles with the odorants of interest and delivery of the odorant /metal nanoparticle mixture to the odor receptors.

It was found that the odorant/metal nanoparticle mixture could be delivered out of an aqueous media and result in significant stimulation of the olfactory neurons as measured by EOG.

6.4b Single cell recording

Rat olfactory epithelium was placed in modified Hanks balanced salt solution without Calcium. The tissue was then transferred to chamber filled with the same buffer. A glass recording electrode (~ 7 mΩ/Tip size ~ - 1μm) which was filled with ICF for control experiments and subsequently with PNC, Zn, Cu, Au, and Ag solutions and secured in an electrode holder, was lowered using a three dimensional micro manipulator, onto the surface of the olfactory epithelium in order to obtain cellular contact.

At this point the tissue was electrically connected to the amplifier through the ground electrode and the recording electrode which was connected to the head stage by means of a holder to enable contact with the solution inside the glass recording electrode. Single cell configuration also yielded dose dependency with incorporation of the metal nanoparticles with the odorants of interest and delivery of the odorant /metal nanoparticle mixture to the odor receptors as of EOG.

6.4c Protocol EOG

Voltage clamp recordings were made with the introduction of odorant puffs as stimuli. Each stimulus lasted for 0.5 seconds and the voltage fluctuated between 63mv to 70 mV. By carefully approaching olfactory sensory neurons with a fine recording glass pipette, it was possible to obtain single-cell voltage-clamp recordings. Recordings were

made after stable contact with the olfactory neuron. Stable electrical recordings were obtained from dissected olfactory epithelia. The mean value for the resting potential, V_o, was -70 (±) 3.6 mV and ranged between –70 to -63 mV. Downward responses indicate inward current.

6.4d Single cell recording

Current clamp recordings were made with the introduction of odorant puffs as stimuli. Recordings were done for 200 seconds with a 20 second intervals between each odor stimulation and with a lasting period of 0.5 seconds. Currents were measured with the Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) in the single-cell voltage-clamp mode. Patch pipettes were fabricated using borosilicate capillaries (WPI, Sarasota, FL) and pulled using a P-87 pipette puller (Sutter Instruments, Novato, CA).

Pipette resistances were 5- 10 MΩ when filled with the internal solution. Voltage-clamped currents were low-pass filtered at 10 kHz. Electrical responses propagated by the application of odor were detected and amplified by the amplifier and the data were relayed from the amplifier to the computer through an analog-to-digital interface Digidata 1322A (Axon Instruments). Acquisition, storage and analysis of data were performed using PClamp 9 software (Axon Instruments). Resting membrane potential was measured as the potential at which the current was zero. Experiments were performed at room temperature.

6.5 Results

Odorant induced olfactory responses were recorded from rapidly isolated rat olfactory epithelia in the presence of crystalline metallic nanoparticles in two different configurations. In the first configuration, the nanoparticles were incorporated with odorant solution (16 M) at different concentrations and OE was stimulated with increasing concentration of this mixture to study the dose dependency. In the second configuration, OE were subjected to odorant mixture alone with nanoparticles filled in the glass electrodes and were allowed to perfuse into OSNs to study the effect of these nanoparticles on the amplitude of olfactory odorant responses with elapsing time. Zn applied to OE either by perfusion or by incorporation with odorant solution attenuated EOG and current responses. Attenuation of EOG and current responses was produced not only by Zn but also by PNC. These attenuations were reversible.

6.5.1 EOG

6.5.1a Perfusion of metals

When introduced with Zn, EOGs produced by odorant solution of interest (1600 μM) were attenuated (fig1). Typical EOGs recovered after the buffer solution with Zn was replaced by buffer alone without Zn. Similar effects were observed with PNC.

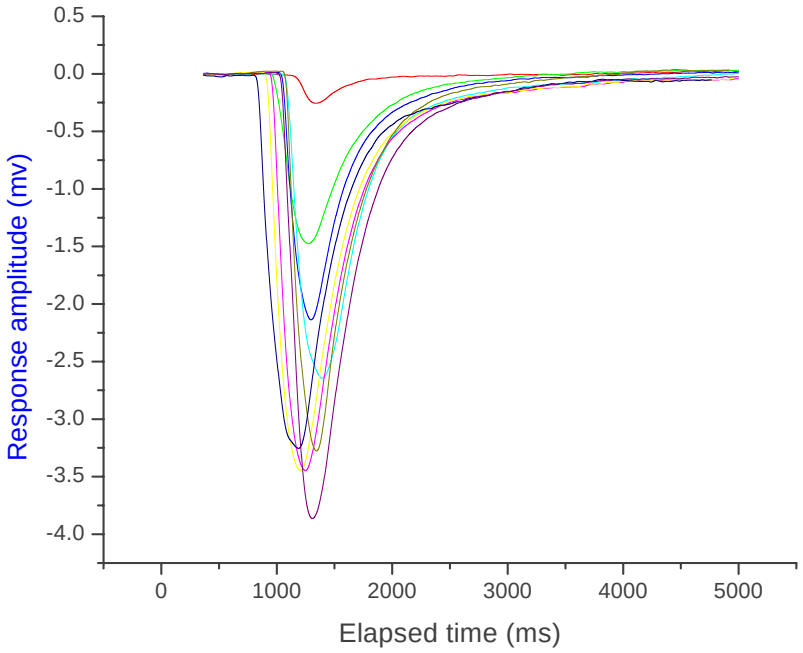

Fig. 1. EOG recordings from a dissected rat olfactory epithelium. Glass micropipette contained Zn was used to diffuse onto the surface of the cilia enhanced substantial odorant response.

Perfusion of Zn nanoparticles and PNC triggered a 10 fold increase in response with elapsing time (fig 1) Perfusion of ECF without Zn (control) resulted in shorter

duration of recording period (~ 20 minutes) and elicited characteristic EOG responses with almost constant or response amplitudes (fig 2).

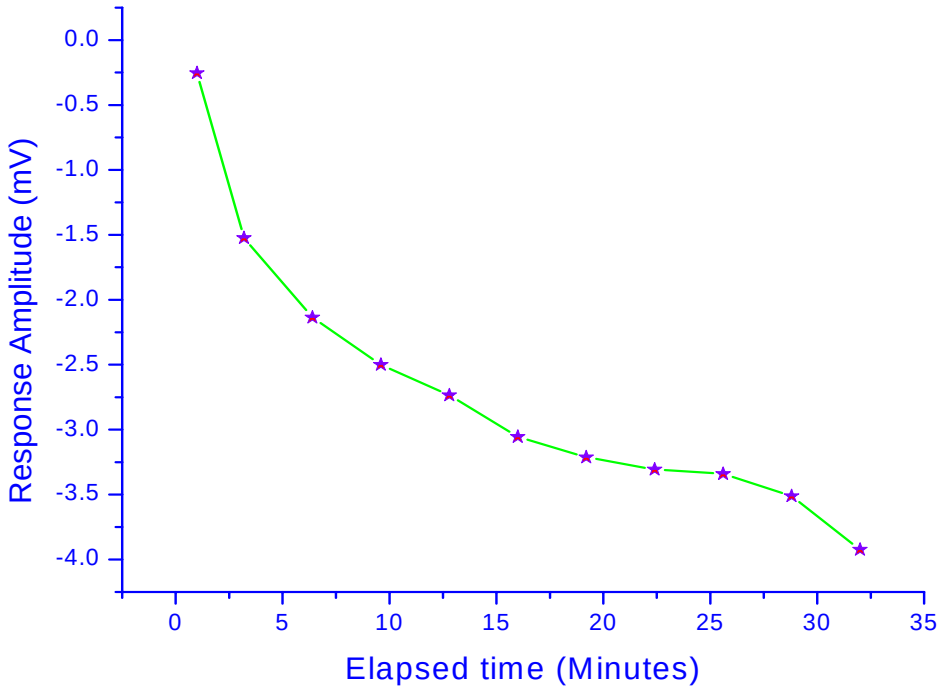

Fig 2 EOG recordings from a dissected rat olfactory epithelium are shown above. Glass electrode contained Zn was used to diffuse onto the surface of the cilia. Graph is plotted as a function of voltage (mV) against time (minutes).

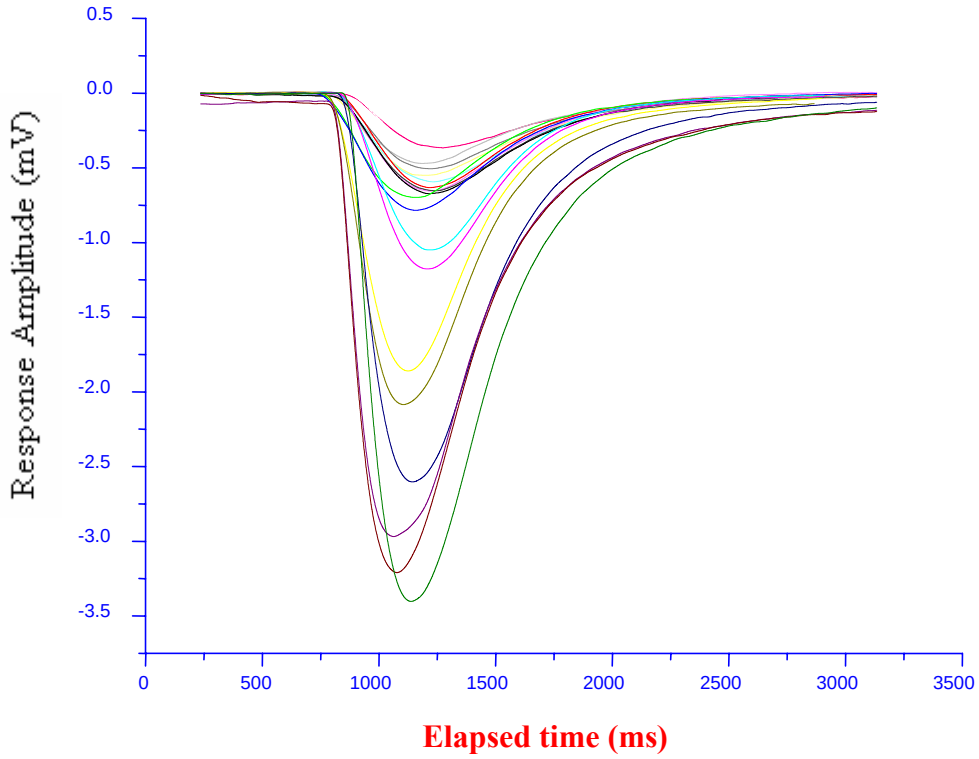

Fig. 3. EOG recordings from a dissected rat olfactory epithelium. Glass electrode contained PNC was used to diffuse onto the surface of the cilia attenuated increased substantial odorant response.

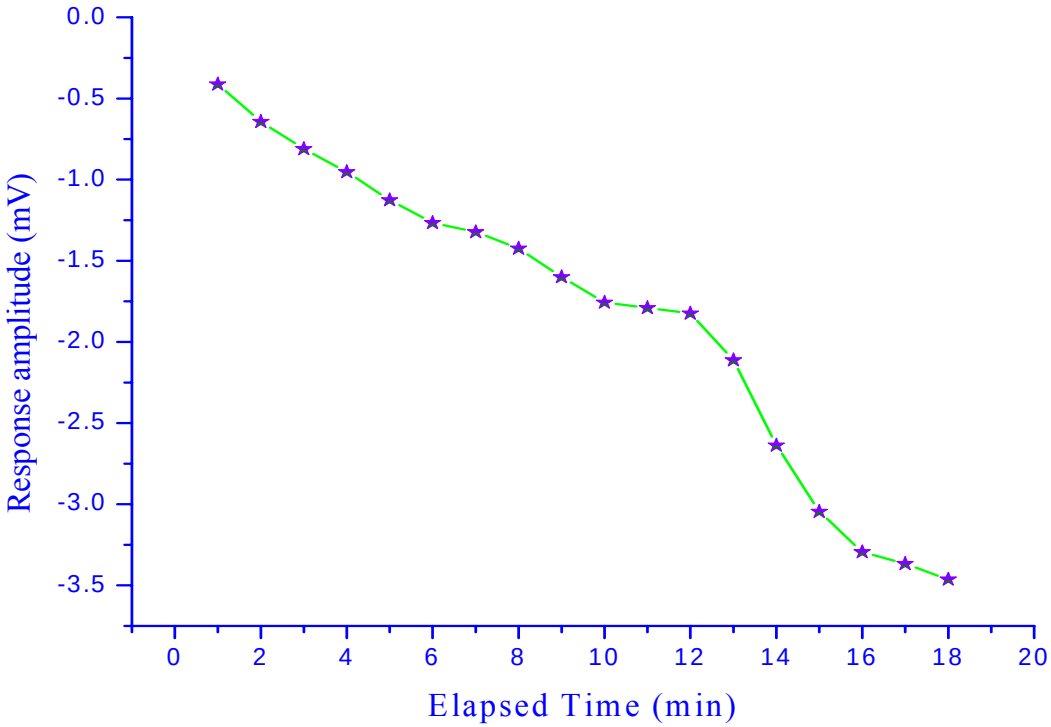

Fig. 4. EOG recordings from a dissected rat olfactory epithelium are shown above. Glass electrode contained PNC was used to diffuse onto the surface of the cilia. Graph was plotted as a function of voltage (mV) against time (minutes). PNC Increased substantial odorant response.

PNC exhibited a prolonged and gradual increase of response amplitude with maximum recording period of 90 minutes. In the case of Zn, the increase in EOG amplitude was prolonged but steep when compared to PNC facilitated EOG responses (fig 4), with a maximum recording period of 50 minutes. With perfusion of Cu an unusually longer recording period of 150 minutes with almost constant response amplitudes was obtained. (Fig 2).

Ag (Fig3) and Au (Fig 4) showed little or no evidence of enhancement. This was marked by tall initial response amplitude, which quickly diminished with distorted EOG's.

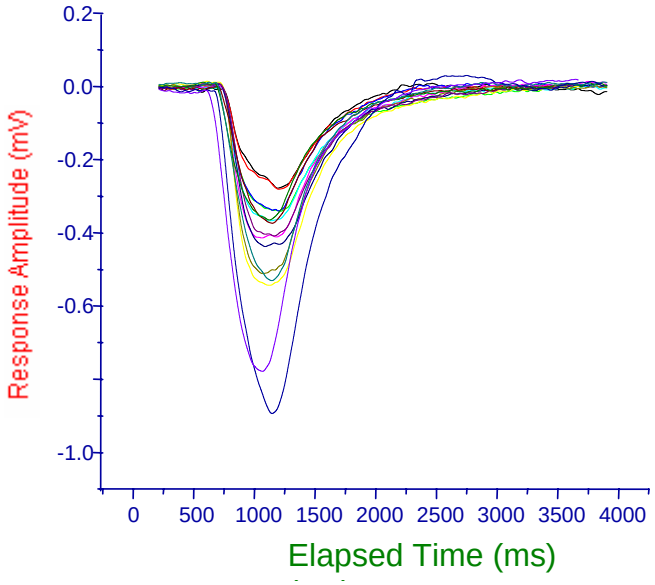

Fig. 5. EOG recordings from a dissected rat olfactory epithelium are shown. Glass Electrode contained Ag was used to diffuse onto the surface of the cilia. Ag displayed initial increment of odorant response which quickly diminished with time.

EOG recordings from a dissected Rat olfactory epithelium are shon. Glass micropipette containing was used to diffuse Gold onto the surface of the cilia. Au displayed initial increments of odorant response which quickly diminished with time.

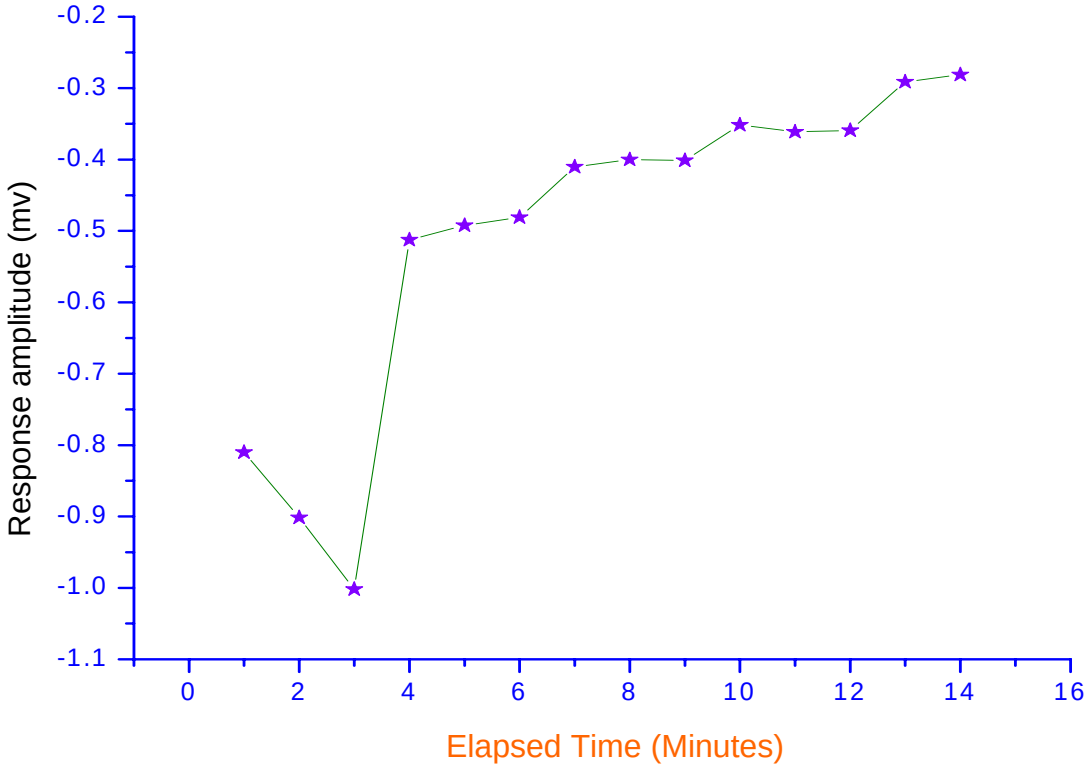

Fig. 6. EOG responses from a dissected rat olfactory epithelium are shown above. Graph was plotted as a function of response amplitude (mV) against time (minutes).

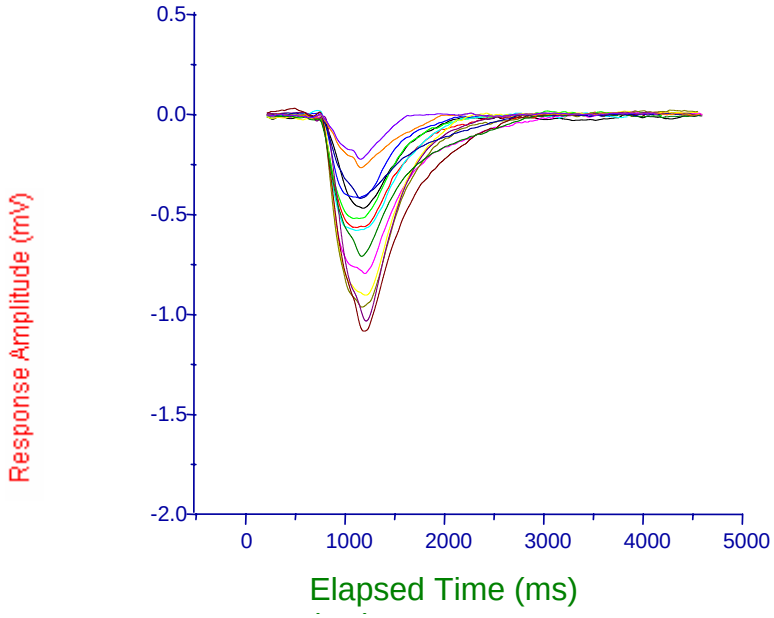

Fig. 7: EOG recordings from a dissected rat olfactory epithelium are shown. Glass electrode contained Au was used to diffuse onto the surface of the cilia. Au displayed initial increment of odorant response which quickly diminished with time as did Ag.

When the electrode filled with Ag solution was introduced to the OE for 15 minutes, EOGs were attenuated. Similar effects were observed with EOGs induced by Au. It was also observed that when Au and Ag were introduced to OE the EOG's been unstable and could not recover after the administration of these metals. (Fig 5)

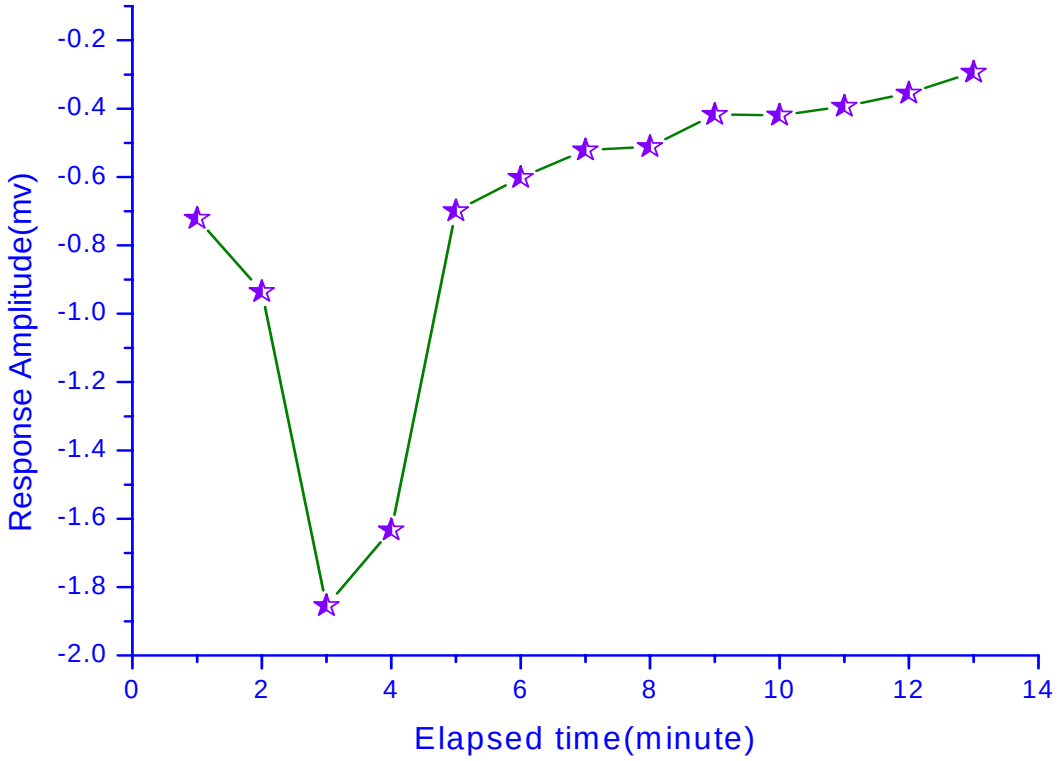

Fig. 8. EOG responses from a dissected rat olfactory epithelium are shown above. Glass micropipette contained Au was used to diffuse onto the surface of the cilia. Graph was plotted as a function of response amplitude (mV) against time (minutes).

6.5.1b Delivery of incorporated metal odorant solution mixture

Nanoparticles incorporated with odorant mixture applied to OE at increasing concentration ratio induced a dose-dependent odorant response in OE (fig 6). Odorant solution mixed with PNC applied to OE at concentration ratio of 5 ml odorant solution to 1, 5, 50, 500 and 1000 µl of PNC induced dose-dependent olfactory responses.

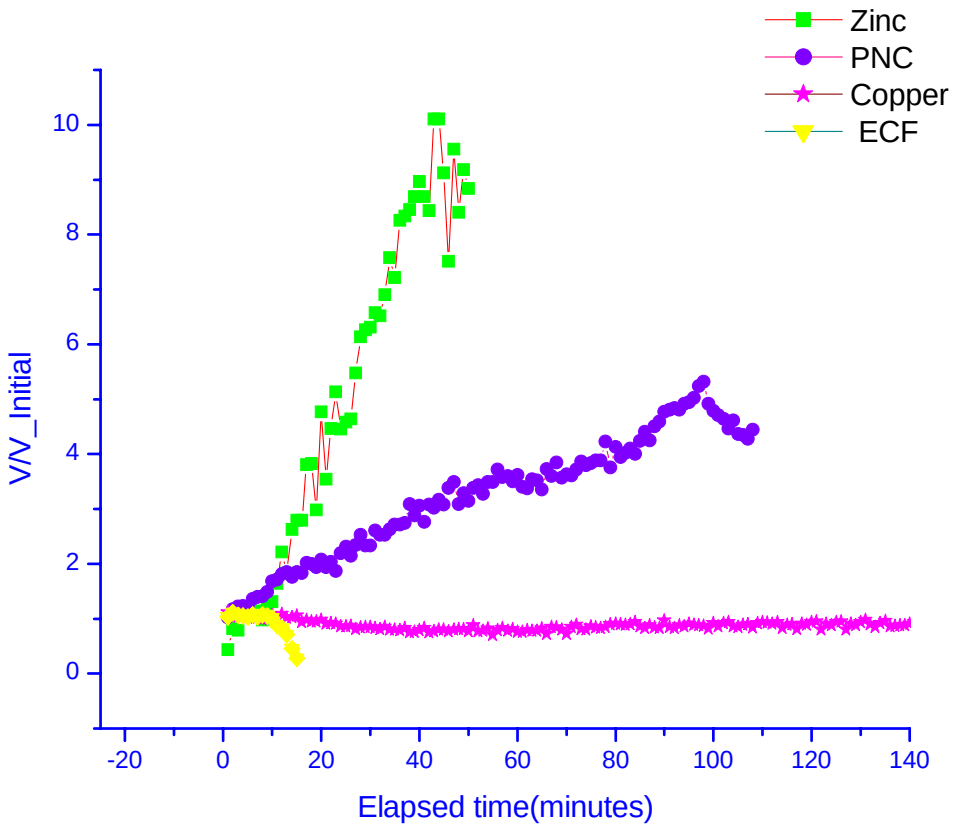

Fig. 9. Graph showing the responses with the perfusion of Zn, PNC, Cu and ECF on OE. With perfusion of copper, the OE responded with almost similar amplitudes up to 140 minutes. With perfusion of Zn, the increase in response amplitude was dramatic and lasted for 50 minutes. PNC displayed gradual increase in response amplitude with time up to 110 minutes. With control ICF the recording period lasted for about 20 minutes with diminishing response amplitudes.

Similarly, odorant solution mixed with Zn applied to OE at similar concentration ratio as above induced a dose-dependent olfactory response. A typical dose dependent relationship was established using increasing concentration of odorant/metal nanoparticle mixture against EOG up to an apparent saturation value at about the ratio of 5ml: 500 µl

in the case odorant mix and PNC and 5ml: 100 µl in the case of odorant mix and Zn. (Fig 7).

6.5.1c Whole cell

Perfusion of metals

Under experimental conditions, perfusion of Zn nanoparticles and PNC triggered a 6 fold increase in response with elapsing time. Under control conditions, odorant stimulation with ICF in the glass electrode did not show marked variation in olfactory responses with elapsing time (Fig 8).

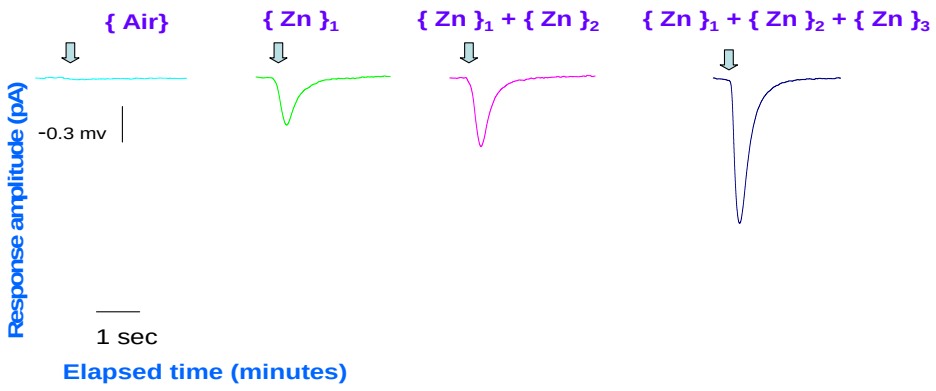

Fig. 10: Dose responses with increasing concentrations of Zinc. Response amplitudes are in the form of current (pA).

Under control conditions, perfusion of ICF resulted in shorter duration of recording (~ 20 minutes) with a constant response amplitude through out the recording period. Perfusion of PNC exhibited prolonged and gradual increase of response

amplitudes with a maximum recording period of ~20 minutes. Perfusion of Zn resulted in a prolonged and steep increase in response amplitude with a maximum recording period of ~ 20 minutes.

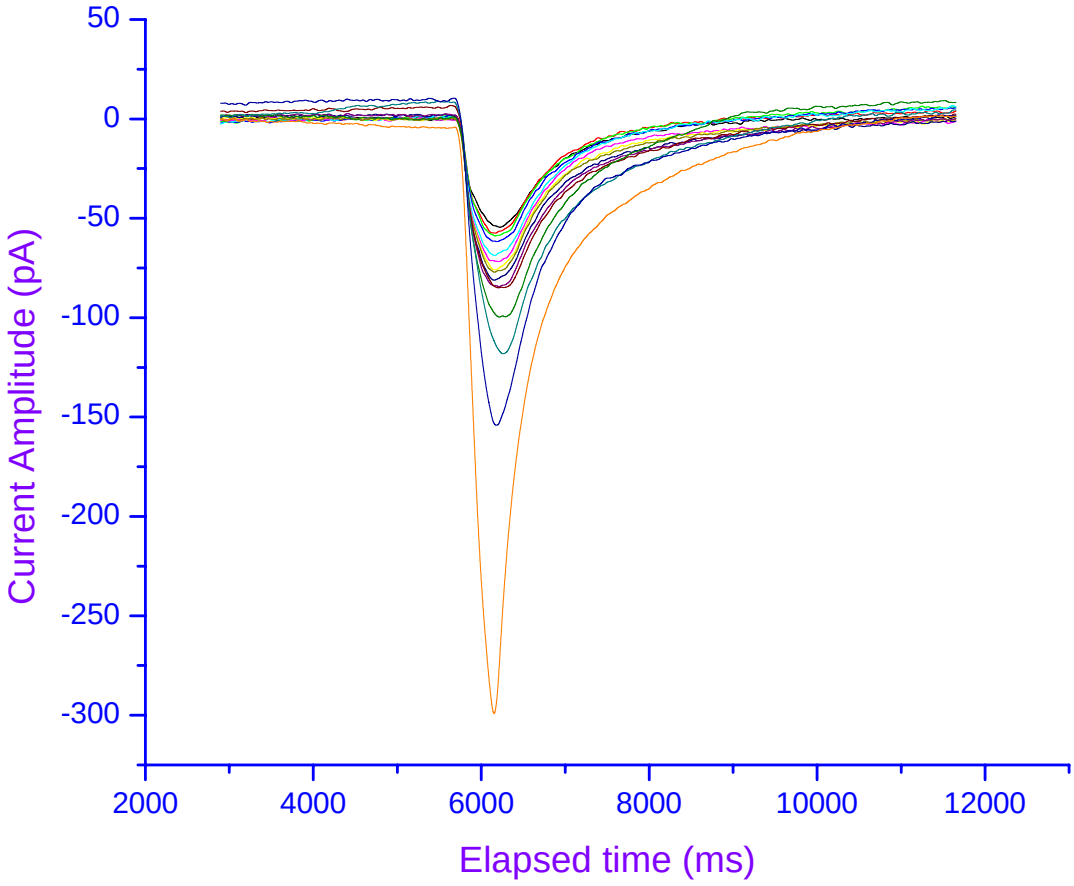

Fig 11 Current responses from single cell perfusion of zinc are shown above. The tip was dipped in ICF

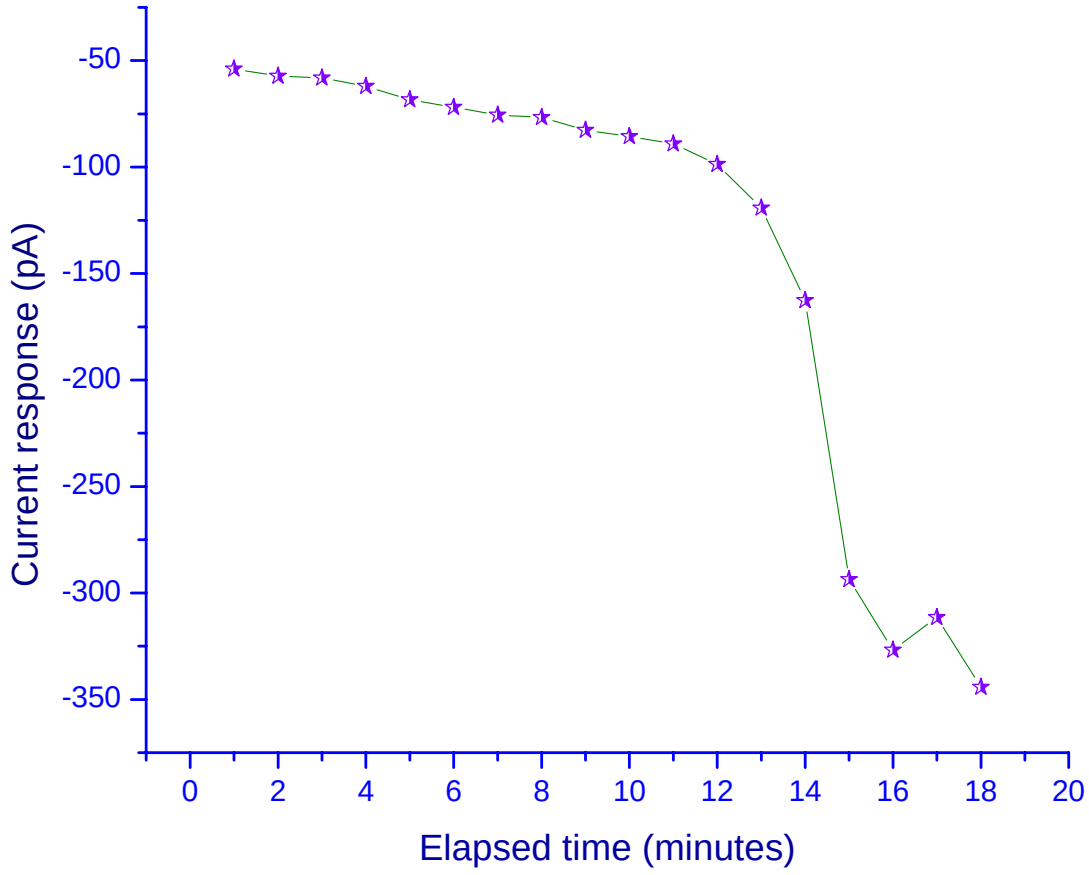

Fig. 12: EOG recordings from a dissected rat olfactory epithelium are shown above. Glass micropipette contained Zn was used to diffuse into the neuron. Graph is plotted as a function of current response (pA) against time (minutes).

6.5.1d Single cell recording with perfusion of PNC

Whole cell recording from a dissected rat olfactory epithelium is shown below, in fig 9. A glass electrode contained PNC was used to facilitate diffusion into the cell. Holding potential was -70mv. PNC increased substantial odorant response (top current). Time course of odorant stimulation is shown below current trace. A mixture of odorant solution was used as a stimulus.

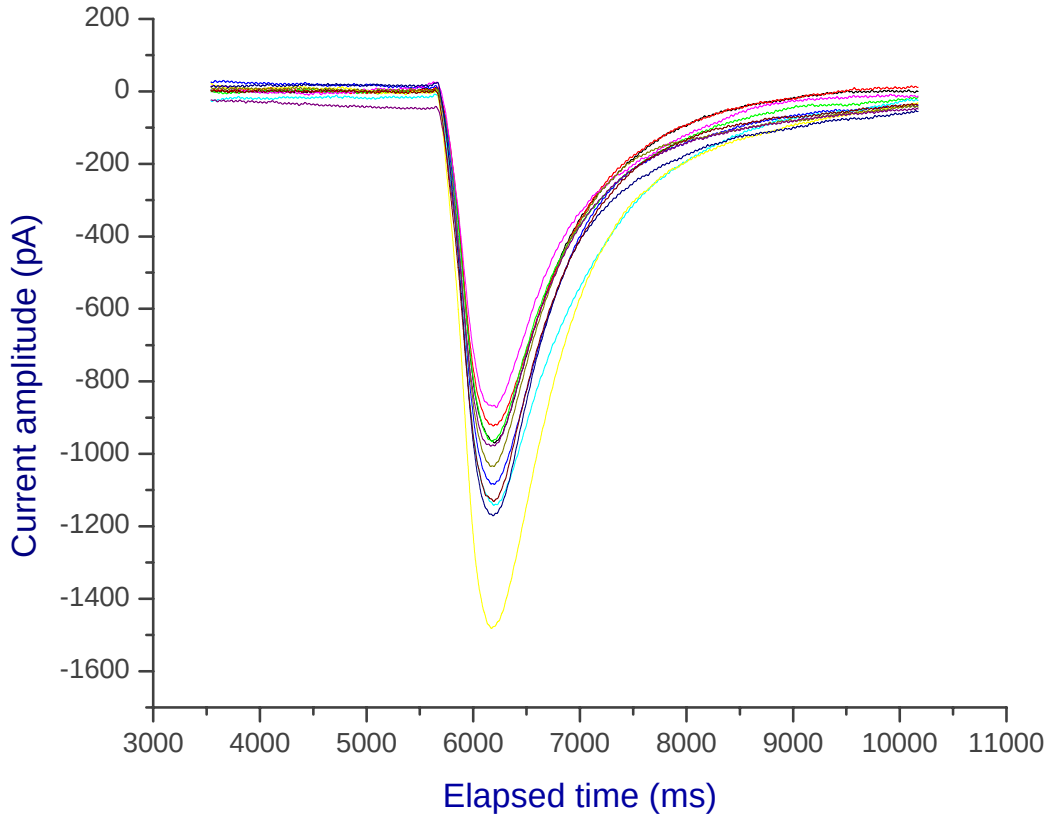

Fig 13 Current responses from single cell perfusion of PNC are shown above. Tip wa dipped in ICF. Duration of recording was 15 minutes. Interval between responses wa 1 minute. Duration of recording was 14 minutes and interval between responses was 1 minute.

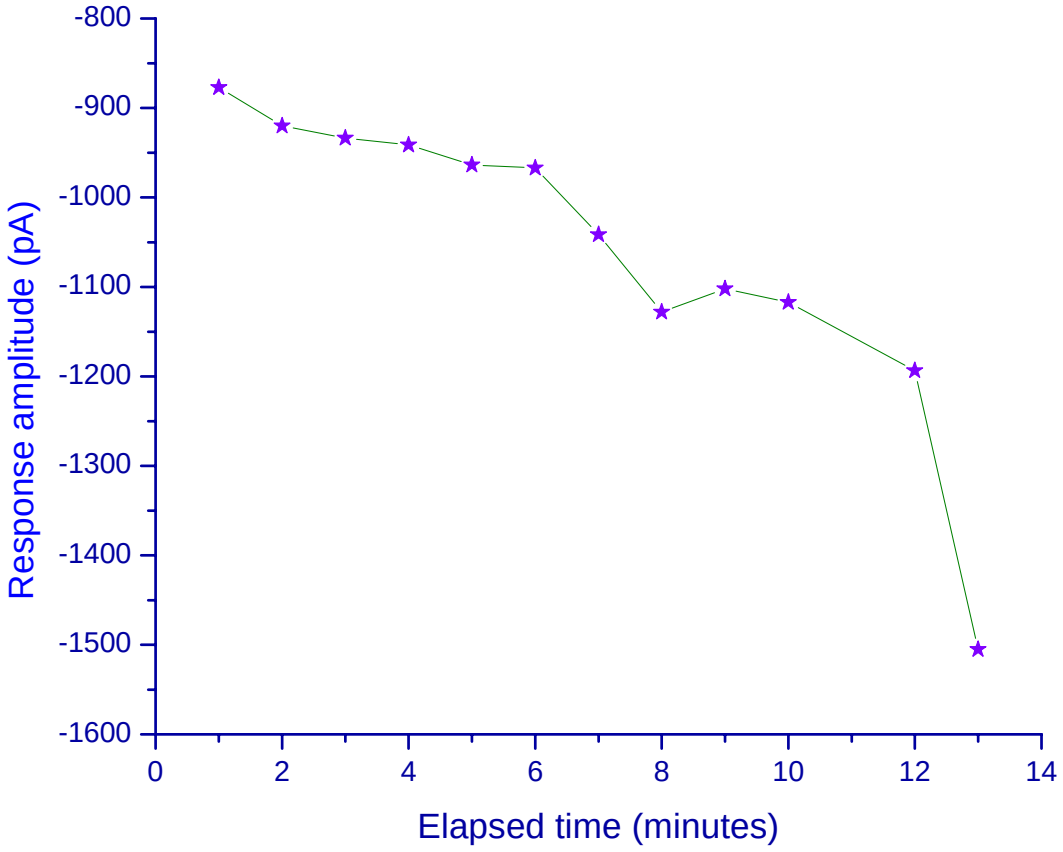

Fig. 14: EOG recordings from a dissected rat olfactory epithelium are shown above. Glass micropipette contained PNC was used to diffuse into the neuron. Graph is plotted as a function of response amplitude in current (pA) against time (minutes).

6.6 Discussion

Zn is an important element in olfaction. However, the role played by Zn in olfaction remains controversial. Zn deficiency causes olfactory disorders (Ishimaru et al

2000), but on the other hand intranasal treatment of zinc sulfate on OE appeared to causes anosmia. Henkin (1975) reported that Zn deficiency caused olfactory loss in humans. Whether Zn is a crucial element in olfaction or a neurotoxin remains a question. We hypothesized that micro molar concentrations Zn^{2+} would increase olfactory sensitivity. This study aimed to establish the acute effects of Zn^{2+} on murine OE by electrophysiological experiments such as EOG and whole cell recordings.

Considerable amount of Zn is present in the olfactory mucosa and may play a role as a neuromodulator, as in the case of hippocampus (Ishimaru et al 2000). In this study, a novel theory of primary olfactory reception has been demonstrated. Our hypothesis here is that Zn molecules are present at the interface of receptors and G Protein and during odorant binding Zn facilitates information transfer from the receptor to G Protein and thereby enhancing olfactory responses. In this tudy we discuss the influence of certain metals particularly Zn during the initial transduction process which takes place in the cilia. Mammalian olfactory system exhibits dual transduction pathways leading to excitation and suppression of odorant response of OE. Much progress has been made in the studies of excitatory pathways of this dual transductory mechanism. Here we discuss yet another mechanism of odorant enhancement in ORNs.

EOG and whole cell olfactory responses from rat olfactory epithelia were examined after the application of selected crystalline metallic nanoparticles derived from biological sources (Proteon Nucleating Centers, PNC) and other compositions. The experiment was carried out with the aim of developing a better understanding of metals in olfaction.

Recent studies on metal influence on olfaction such as, electron tunneling in olfactory receptors (Turin 1996), shuttlecock movement of the receptor protein during odorant binding (Wang et al 2001) and vital roles played by Zn in central and peripheral nervous system lead to realize the strong link between Zn and olfaction. Luca Turin proposed a molecular model in which there is a Zn binding site in both the odorant receptors and the G-protein. If the Zn binding motif on the olfactory receptor is involved in docking to the olfactory G_{olf}-protein and thus leading to the formation of a disulfide bridge between receptor and G_{olf} to the other half of a zinc coordination site. Turin (1996) proposed a similar mechanism in olfaction

Wang et al (2001) observed that in OR's, the loops between the putative 4^{th} and 5^{th} transmembrane helices are unusually long and hydrophobic which lead them to a hypothesis concerning the structural change of the receptor during odorant binding. The hypothetical "shuttlecock" mechanism for metal ion- aided odorant recognition was based on the two different stable conformations for the OR seven-helix structure. According to Wang et al, initially, the OR is a seven-helix structure conformation in the currently accepted form. Upon metal binding, the anionic charge of the 4–5 loops is neutralized, thus allowing the loop to become helical and infiltrate into the membrane. They believe this conformation, is the active form of metal ion-containing ORs. In our study, the metallic nanoparticle compositions enhanced the EOG potentials elicited by a mixture of odorants by up to 10 fold and single cell current amplitudes by up to 6 fold. With perfusion of Cu, the EOG amplitudes did not vary but were constant for an unusually

prolonged period of time, a unique feature observed in our experiments. Cu appears to facilitate long lasting and stabilized olfactory response.

Winberg et al (1992) studied the effect of inorganic Cu on OE of Atlantic salmon (Salmo salar L) EOG , by irrigating the OE with aqueous copper solutions in increasing concentrations (0.2 to 9.7 μM) of free Cu^{2+}. The amplitudes and form of the EOGs increased drastically with increasing Cu^{2+} concentrations. The study indicates the excitatory effects of Cu ions on the transduction mechanisms of the olfactory receptor cells.

In this study, perfusion of Zn and PNC caused the odorant response amplitudes to increase steadily and the effect began to diminish after a period of 60 minutes. The decrease in EOG amplitude may be due to the experimental condition when the OE begins to wither. Perfusion of metals like Au and Ag exhibited strong responses in the initial stage of the recording period which quickly disappeared with distorted EOG recordings. This might be attributable to the damage caused by these metals to the neuron. Under control conditions, odorant induced stimulation with ECF in the glass electrode did not show marked variation in olfactory responses with elapsing time.

Metal compositions also exhibited dose-dependent olfactory responses. The dose-response curves indicate that OE responds to mixture of odorant and metals up to an apparent saturation state. However, mixture of odorant and Cu with similar dilution as of Zn elicited almost constant amplitude of odorant responses. Also, under control conditions odorant induced stimulation alone in the absence of metals did not show marked variation in olfactory responses with elapsing time.

An increase in odorant induced current amplitude was also observed when nanoparticles were filled in glass electrodes and perfusion took place over time. A progressive increase in the diffusion rate may have triggered an increase in the amplitude of the responses. Perfusion of Zn and PNC resulted in prolonged increase in response amplitudes with maximum recording period of ~20 minutes. However this recording period was much shorter when compared to much longer recording period elicited from EOG. The shorter recording period in single cell recording may be attributed to the shortcoming of single cell recording methods

The enhancing effect of metal induced odorant responses may be due the binding of Zn to G protein and may have contributed to the progressive increase in the odorant induced olfactory response amplitudes by enhancing its effect on adenyl cyclase (AC). The enhancement of odorant induced current by PNC and Zn indicates the positive effect of the nanoparticles on AC.

EOG studies by Ishimaru et al (2000) revealed the acute effects of Zn^{2+} on olfactory epithelium of bullfrog (Rana catesbiana). When the buffer solution without Zn^{2+} applied on the ciliated side OE was replaced by a solution containing Zn^{2+} (25 μM), EOGs produced by n-amyl acetate (200 μM) were attenuated. EOGs recovered after the buffer solution with Zn^{2+} was washed out and replaced by solution without Zn^{2+}. Similar effects were observed with another odorant, menthone (10 μM). When the Zn concentration was within 10 μM, effect of Zn was unstable and EOG could not recover at 50 μM.. Zn^{2+} (25μM) charged ciliated surface of the olfactory mucosa attenuated EOG's, which were produced not only by odorants but also by forskolin and IBMX. Forskolin

and IBMX increase the c-AMP concentration in intra-olfactory cells. This indicates that Zn^{++} may block the c-AMP dependent transduction channels of the olfactory cells. Olfactory bulb of rat treated with intranasal administration of cerebrolysin and zinc sulfate showed no significant elevation of zinc (Kudrin et al 2004). Consecutive intranasal administration of zinc and cerebrolysin for 10 days resulted in 4.5-fold increase of zinc in the olfactory bulbs. The study suggests that consecutive administration of Zn and neuroprotective drugs could be applied in treatment of some metal-related brain disorders (Kudrin et al 2004).

Studies show that the direct binding of Zn^{2+} to proteins like β-amyloid in the case of Alzheimer's disease, ά-synuclein in Parkinson's disease, superoxide dismutase in amyotrophic lateral sclerosis, and prion in spongiform encephalopathies, results in either the excess or lack of Zn^{2+} binding, and thus promoting these protein misfolding disorders**. (**Stojanovic et al 2004). In the case of spongiform encephalopathies and Alzheimer's disease, Zn^{2+} promotes aggregation of the highly fibrillogenic prion peptide, PrP106–126 (Jobling 2001), and the β-amyloid protein (Atwood et al 1998) into amyloidogenic aggregates. But in superoxide dismutase protein, reduction of Zn^{2+} affinity results in lack of protein activity (Crow et al 1997). Stojanovic et al (2004) observed that in rhodopsin, receptor destabilization through the addition of increased concentrations of Zn^{2+} was because of the binding to Zn^{2+} coordination site in the transmembrane domain and plays a critical physiological role in rhodopsin stabilization in the dark.

The studies confirm that the high-affinity metal coordination site in the transmembrane domain was selective for Zn^{2+}. Studies also suggest that high

concentrations of either Zn^{2+} or Cu^{2+} can destabilize rhodopsin protein (Del Valle et al 2003).

According to Bresink et al (1996) Zn changes AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) receptor properties in rat cortical membranes. Zn in HCL buffer significantly increased the specific binding of [3H] AMPA. At equilibrium [3H], AMPA binding is reached with faster kinetics in the presence of Zn.

According to Tjalve (1996) Mercury is known to alter olfaction and olfactory-related behavior in fish. Tjalve (1999) studied the mechanism of uptake and transport of metals like manganese, cadmium, nickel and mercury from the OE to the rest of the olfactory system including areas of brain. (Neurotoxicology 1999 Apr-Jun;20(2-3):181-95.Uptake of metals in the brain via olfactory pathways, Tjalve H, Henriksson J).

Takagi et al (1969) reported that Zn^{2+} solution (88.4 mM) drastically attenuated EOG which was not recoverable. This may be due to the high concentration of Zn used in his experiments. Zn^{2+} is neurotoxic (Yokoyama 1986 and Weiss 1993) and exhibits blocking effects on ion channels in neurons of central nervous system. Zn^{2+} blocks N-methyl-D-aspartate (NMDA) receptor channels (Gray et al 1987 and Williams et al 1997), and voltage gated calcium channels (Büsselberg et al 1994). The cyclic nucleotide channels of the olfactory cilia, activated by c-AMP are modulated by μM concentrations of Ca^{2+} or Mg^{2+} (Kurahashi et al 1989, Kurahashi 1990, and Nakamura et al 1987). The effective concentration of Zn^{2+} is much lower than Ca^{2+} or Mg^{2+}.Also zinc is reportedly released from secretary granules into the surface of the olfactory mucosa (Ishii 1999).

The neuromodulatory function of Zn in the hippocampus [Ishii 1999] correlates with the Zn^{2+} modulation of olfactory transduction channels, and it is possible that Zn may play a vital role in the olfactory transductory system.

6.7 Conclusions

Presence of micro Molar concentrations of Zn^{2+} attenuates EOG of rat OE and may behave as one of the modulators in olfactory reception. Olfactory stimulation of rat OE was measured using EOG. The metallic nanoparticles enhanced the EOG potentials and single cell current responses elicited by a mixture of odorants. Also the method for incorporation of the metal nanoparticles with the odorants and delivery of the odorant /metal nanoparticle mixture to the odor receptors resulted in significant stimulation of the olfactory neurons. Odorant-induced currents suggest that metallic nanoparticles are physiologically significant for olfactory signaling.

The behavior we observed in rat olfactory tissue is valuable because it reveals one of the important properties of the olfactory system. The data will help us to better understand the dynamics of olfactory enhancement and employ it in constructing a practical olfactory response model. The practical applications of this unique observation of enhancement of olfactory receptor neuronal responses to odorants are significant. Aside from providing indirect evidence for the involvement of a metal ion in the transduction process, metal binding could also be useful in fragrance design if it were possible to build into the molecule so as to increase odorant potency.

6.8 References

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7. CONCLUSIONS

The heterotrimeric G-protein G_{olf} links cell surface receptors to the activation of adenylyl cyclases and cAMP production. Here we report that G_i modulate cAMP production by odorant-stimulated olfactory epithelium membranes, where the Gsα family member $G_{olf\alpha}$ links odorant receptors to adenylyl cyclase activation. G_i reduces odorant elicited cAMP production by acting as an inhibitor of adenylyl cyclase type III, the predominant adenylyl cyclase isoform in olfactory neurons. Furthermore, whole cell voltage clamp recordings of odorant stimulated olfactory neurons indicate that endogenous G_i negatively regulates odorant-evoked intracellular signaling. These results reveal a new mechanism for controlling the activities of adenylyl cyclases, which likely contributes to the exquisite ability of olfactory neurons to discriminate odors.

Long-term culture of OE and OB tissues is possible and promising as a model for electrophysiological studies. Olfactory receptor neurons responded to odorants in culture least 36 days .The main Factor known to enhance ORN survival include physical contact with olfactory bulb tissue .Reasons for decline in ORN population include death of neurons after severing axons during dissection, lack of proper growth factors for basal cell proliferation and differentiation or ORN maturation and survival, lack of proper extra cellular ligands, failure of OE and OB to maintain contact Chuah (19). It is reasonable to expect ORNs and OBNs to survive in vitro, and even to expect them to engage in complex synaptic arrangements similar to those found in vivo.

Dissociated rat and mouse OB develop synaptic structures suggestive of reciprocal dendro-dendritic synapses, and dissociated rat OB also contains GAD^{+} neurons Fracek, 1994.

Adult zinc deficiency reportedly leads to degeneration of the olfactory epithelium in the rat. Human zinc deficiency can cause reduced olfactory sensitivity. We report here on the effects of zinc olfactory responses. With Zn as an odorant and with dialysis of Zinc into the olfactory neuron Zinc appeared to increase olfactory responses .In contrast Copper did not enhance the odorant responses. However it appeared that exposure to copper resulted in a prolonged period of responds time. Zinc is an essential component of various proteins and is an important modulator in the mammalian central nervous system (CNS). Zinc deficiency impairs neuro- and immunoactivity of the mammalian organisms.