Characterization of Seven Transmembrane Domain Receptor Gene Expression in Olfactory Receptor Neurons.

Kathryn Medler
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/6751

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
CHARACTERIZATION OF SEVEN TRANSMEMBRANE DOMAIN RECEPTOR GENE EXPRESSION IN OLFACTORY RECEPTOR NEURONS

A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biological Sciences

by
Kathryn Medler
B.S., Texas A&M University, 1989
M.S., San Diego State University, 1992
August, 1998

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ACKNOWLEDGMENTS

I wish to thank my husband and son, Scott and Peyton Medler, for their support and understanding over the last four years as I pursued my education. I also wish to thank Rick Bruch for all his mentoring, advising, and general pushing needed to help keep me motivated to work through problems as they arose. I thank Hang Tran, who’s reliable assistance with every aspect of this work enabled me to accomplish a great deal of work in a short time period. I also thank all the other people I worked with in the lab who assisted me with my work, either by advising me with problems or by helping me get the work done: Michele Rankin, Michael Moore, Sparky Guidry, and Rebecca Alvania. I also wish to thank Scott Medler for photographic assistance, Jim Parker for electrophysiological analysis, Judy Buchholz for sectioning tissue, Dr. John Lynn for use of his microscope, Dr. Tim Gilbertson for assistance with micromanipulators, and Dr. Jackie Stevens for assistance with image analysis.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ................................................................. ii

LIST OF FIGURES ................................................................. v

LIST OF ABBREVIATIONS ....................................................... vii

ABSTRACT .................................................................................... ix

CHAPTER 1. INTRODUCTION .............................................................. 1
  1.1 Odorant Receptor Gene Expression in the Olfactory Epithelium  ............................................. 1
  1.2 Other Receptors Expressed in Olfactory Neurons ............................................................... 12
  1.3 Receptor Regulation ............................................................................................................. 15
  1.4 Dissertation Specific Aims ..................................................................................................... 18

CHAPTER 2. ODORANT RECEPTOR EXPRESSION IN SINGLE OLFATORY RECEPTOR NEURONS AND TASTE BUDS FROM CHANNEL CATFISH, ICTALURUS PUNCTATUS .............................................. 22
  2.1 Introduction ....................................................................................................................... 22
  2.2 Materials and Methods ....................................................................................................... 23
  2.3 Results ............................................................................................................................... 31
  2.4 Discussion ........................................................................................................................... 47

CHAPTER 3. METABOTROPIC GLUTAMATE RECEPTOR EXPRESSION IN OLFATORY RECEPTOR NEURONS FROM THE CHANNEL CATFISH, ICTALURUS PUNCTATUS .............................................. 54
  3.1 Introduction ....................................................................................................................... 54
  3.2 Materials and Methods ....................................................................................................... 56
  3.3 Results ............................................................................................................................... 63
  3.4 Discussion ........................................................................................................................... 74
  3.5 Endnote .............................................................................................................................. 81

CHAPTER 4. PHOSPHORYLATION BY PROTEIN KINASE C OF ODORANT RECEPTORS AND METABOTROPIC GLUTAMATE RECEPTORS .............................................................................. 82
  4.1 Introduction ....................................................................................................................... 82
  4.2 Materials and Methods ....................................................................................................... 85
  4.3 Results ............................................................................................................................... 92
  4.4 Discussion ........................................................................................................................... 101
LIST OF FIGURES

2.1 Photomicrograph of dissociated cells from olfactory rosettes. .......... 26

2.2 PCR products from single neurons digested with frequent cutting endonucleases. ................................................................. 33

2.3 Sequence of PCR products of putative odorant receptors amplified from 3 individual olfactory receptor neurons. ....................... 34

2.4 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 35

2.5 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 37

2.6 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 38

2.7 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 39

2.8 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 40

2.9 Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron. ......................... 41

2.10 Sequence of PCR products of putative odorant receptors amplified from catfish barbel epithelium. .......................................... 45

2.11 Southern blot analysis of PCR products. ........................................ 46

3.1 Sequence of mGluR PCR products amplified from olfactory epithelium. . 65

3.2 Sequence of PCR products of putative odorant receptors amplified from olfactory receptor neurons that were coexpressed with the mGluRs. . 67

3.3 Southern blots of PCR products obtained from individual olfactory receptor neurons. ................................................................. 69

3.4 Micrographs of the localization of mGluRs by immunocytochemistry. . 70

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
3.5 Effect of the glutamate antagonists, S-4CPG and MCCG, on integrated olfactory receptor responses to L-glutamate and L-methionine. 72

3.6 Graphical representation of the calculated peak areas for glutamate and methionine responses in the presence of mGluR antagonists. 73

4.1 Vector map of leader sequence from the pTrcHis vector. 93

4.2 Mutations of consensus phosphorylation sites for odorant receptor and glutamate receptor. 95

4.3 Primer sets used to amplify hydrophilic loop domain containing consensus phosphorylation sites in odorant receptor and mGluR1. 98

4.4 Autoradiographs of receptor phosphorylation assays. 100
LIST OF ABBREVIATIONS

7TMD-7 transmembrane domain
βARK-beta adrenergic receptor kinase
bp-base pair
cAMP-cyclic adenosine monophosphate
cDNA-complementary deoxyribonucleic acid
DTT-dithiothreitol
G protein-guanine nucleotide binding regulatory protein
GRK-G-protein coupled receptor kinase
Gs-stimulatory G protein
IP3-inositol trisphosphate
IPTG-isopropylthio-β-D-galactoside
MCCG-α-Methyl-L-CCG l/(2S,3S,4S)-2-Methyl-2-(carboxycyclopropyl) glycine
mGluR-metabotropic glutamate receptor
mRNA-messenger ribonucleic acid
MS-222-methanesulfonate salt
NMDA-N-methyl-D-aspartate
PCR-polymerase chain reaction
PIP2-phosphatidylinositol 4,5-bisphosphate
PKA-protein kinase A
PKC-protein kinase C
PLC-phospholipase C
PMSF-phenylmethylsulfonyl fluoride
POLY A+-RNA-poly adenosine ribonucleic acid
RT-PCR-reverse transcription-polymerase chain reaction
S-4CPG-(S)-4-carboxyphenylglycine
SDS-sodium dodecyl sulfate
ABSTRACT

The expression of 7 transmembrane domain receptors in olfactory receptor neurons of the channel catfish, Ictalurus punctatus was characterized. Due to disagreements in the literature as to how many odorant receptor genes are expressed in olfactory neurons, this study directly measured the number of odorant receptor gene transcripts expressed in single olfactory neurons. Individual olfactory receptor neurons can express more than one receptor, with some neurons expressing at least 3 to 4 receptors. These findings correlate with electrophysiological evidence but disagree with conclusions based on in situ hybridization. Receptors similar to odorant receptors are also expressed in taste buds, indicating a possible role for these receptors as detectors of taste stimuli. The odorant receptors may belong to a larger group of chemoreceptors that function as detectors of various signals depending on the cell in which they are expressed.

The expression of metabotropic glutamate receptors (mGluR) in olfactory receptor neurons was also characterized. Two subtypes of these receptors, mGluR1 and mGluR3, were found in olfactory receptor neurons. These receptors were coexpressed with each other and with odorant receptors. Immunocytochemical analysis determined that these receptors were localized in the dendritic knobs and cilia of the neurons and electrophysiological evidence indicated that these receptors affected the response to the glutamate odorant.
These findings support the hypothesis that mGluRs may have a function in olfaction.

The last aim of this dissertation characterized the phosphorylation of the odorant and mGluRs by protein kinase C (PKC). An in vitro assay was used to determine if the PKCs known to be expressed in olfactory receptor neurons, PKCβ and PKCδ, phosphorylate a consensus site found on both receptors. It was shown that mGluRs were phosphorylated by PKCβ and PKCδ, with higher phosphorylation occurring by PKCβ. PKC may thus function to desensitize mGluRs in vivo. The consensus site on the odorant receptor was only phosphorylated by PKCδ. This site is located on extracellular loop 2 of the odorant receptor and when phosphorylated may function as a targeting signal during processing of the receptor.
CHAPTER ONE. INTRODUCTION

1.1 ODORANT RECEPTOR GENE EXPRESSION IN THE OLFACTORY EPITHELIUM

The process of transducing an olfactory stimulus from the environment to the internal components of olfactory receptor neurons has been the focus of many studies. It is currently thought that odorants bind to receptors on the cilia of the olfactory receptor neurons (Rhein & Cagan, 1980). This binding results in the activation of heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) which increases second messenger levels in the cell (Bruch & Kalinoski, 1987). These second messengers, either cyclic adenosine monophosphate (cAMP) or inositol trisphosphate (IP₃), are believed to gate nonselective cation channels which results in an increase of ion levels within the cell. Changes in ion levels within the cell alter the membrane potential and send a signal to the olfactory bulb that olfactory stimuli have been encountered.

Intense research in olfaction has focused on the characterization of the odorant receptors. Initial studies characterized the site of interaction for olfactory stimuli on the olfactory receptor neuron. Several studies showed that fish serve as good models to study the characteristics of receptor/ligand binding. In contrast to mammals that detect volatile hydrophobic olfactory stimuli, fish respond to hydrophilic amino acids. Since amino acids are odorant ligands as well as non-volatile, it is possible to measure odorant receptor
binding in fish in a quantifiable, repeatable assay. Several species including rainbow trout (Cagan & Zeiger, 1978; Rhein & Cagan, 1980; Rhein & Cagan, 1983), salmon (Rehnberg & Schreck, 1986), and catfish (Bruch & Rulli, 1988), have been used to characterize the binding of olfactory stimuli to receptors. These studies have found similar binding properties between fish species.

Early studies in rainbow trout biochemically characterized the binding of several known odorants to isolated membrane preparations from olfactory tissue (Cagan & Zeiger, 1978). The sedimentable fraction which was enriched in plasma membranes was the site of most ligand binding. Using this fraction, Cagan & Zeiger (1978) showed that multiple types of odorant binding sites were present but that not all sites were exclusive for a single stimulus. Specifically, the authors characterized several binding sites by the amino acid ligand used as a stimulus. Some sites were apparently specific for a single stimulus such as a specific site for L-lysine, while other sites were common for multiple stimuli, such as the TSA site that could bind L-threonine, L-serine, and L-alanine (Cagan & Zeiger, 1978). Later work biochemically determined that the olfactory cilia were the location of the odorant recognition sites (Rhein & Cagan, 1980). With the clarification that olfactory cilia contain binding sites for odorant stimuli (Rhein & Cagan, 1980), it was possible to begin characterizing odorant binding biochemically and electrophysiologically, even though the molecular structure of the receptors was unknown.
The properties of binding sites were characterized (Rehnberg & Schreck, 1986; Rhein & Cagan, 1983) and competitive binding experiments showed that the ability of odorants, in this case amino acids, to bind to specific binding sites depended on the molecular size and charge of the odorant (Rehnberg & Schreck, 1986). Rehnberg & Schreck (1986) also related behaviors of the salmon to specific odorants and found that amino acids, such as serine and alanine, believed to compete for the same binding sites, all induced a similar avoidance behavior, while other amino acids did not. These data further supported the hypothesis that while there are multiple odorant binding sites, structurally similar ligands can bind to the same receptor site and essentially act as identical odors (Rehnberg & Schreck, 1986).

Further work characterizing the L-alanine receptor in catfish correlated receptor binding specificity with stimulus specificity. Competitive binding studies found that amino acids with structural features similar to alanine effectively competed for the L-alanine binding site. Amino acids that had divergent structures did not effectively compete with L-alanine for its binding sites (Bruch & Rulli, 1988). These structural differences correlated with different receptor sites that had been characterized electrophysiologically and included receptor sites for acidic amino acids (A), basic amino acids (B), hydrophilic neutral amino acids with short side chains (SCN), and the more hydrophobic amino acids that contain long side chains (LCN). These groups of amino acids did not significantly cross-adapt with each other in vivo, suggesting
that the electrophysiological responses obtained from each stimulus group were due to different receptor and/or transduction pathways (Caprio & Byrd, 1984). L-alanine was classified as a SCN amino acid (Caprio & Byrd, 1984) and it was found that all other SCN amino acids tested were able to significantly compete for the alanine binding site. Most derivatives of L-alanine as well as D-alanine were not able to effectively compete for the L-alanine binding site, suggesting that the steric structure of the molecule determines its binding (Bruch & Rulli, 1988). Bruch & Kalinoski (1987) also showed that L-alanine and L-arginine binding affinity was decreased in the presence of guanine nucleotides, indicating that the binding sites were linked to a GTP-binding regulatory protein, further supporting the hypothesis that a receptor is involved in ligand binding. The amino acid competitive binding affinities (Bruch & Rulli, 1988) generally correlated with electrophysiological work (Caprio & Byrd, 1984) in catfish and indicated that there are selective receptors for amino acids that discriminate between ligands due to differences in their structure and/or charge. This correlates with findings in other fish systems as well (Rehnberg & Schreck, 1986; Rhein & Cagan, 1983) indicating that ligand specificity may be a general characteristic of amino acids receptors that act as odorant receptors in fish.

Despite the evidence demonstrating the likely presence of odorant receptors, there was no molecular characterization or structural definition of the actual receptor until Buck & Axel (1991) published their study on rats. It is
currently accepted that the olfactory receptors characterized in their study (Buck & Axel, 1991) are the actual odorant receptors that transduce the odor signal from the environment into the cell. However, it must be noted that until direct evidence indicates that these receptors actually bind odors as their primary ligand, the role of these receptors must remain putative. Evidence is beginning to emerge from studies on Caenorhabditis elegans (Sengupta et al., 1996; Zhang et al., 1997) which have correlated a specific ligand to a specific receptor. Initially, Sengupta et al. (1996) found that odr-10 mutants were deficient in their chemotactic ability to the odorant diacetyl and that expression of the wild type odr-10 cDNA restored diacetyl sensitivity in a mutant that had lost its ability to respond to several odorants. odr-10 encodes a seven transmembrane domain receptor that is more similar to vertebrate olfactory receptors than to any other G protein coupled receptor (Sengupta et al., 1996). In later studies, odr-10 was expressed in a heterologous system and it was found that human cells expressing odr-10 on their surfaces had a transient rise in intracellular calcium levels after the application of diacetyl. Other volatile chemicals similar to diacetyl did not evoke a calcium response but two precursors to diacetyl, pyruvate and citrate, did elicit a response. These results indicated that the odr-10 gene does encode an odorant receptor specific to diacetyl (Zhang et al., 1997). Expression of ODR10 from C. elegans and an odorant receptor from zebrafish has also been performed in vertebrate kidney cells (HEK293 cells). Odorant stimulation of these expressed receptors...
resulted in a transient increase in intracellular calcium levels, indicating that these receptors were functioning as odorant receptors (Wellerdieck et al., 1997).

One of the first studies to characterize the function of the odorant receptors expressed two odorant receptors from the rat in the baculovirus-Sf9 cell system. Cells expressing the olfactory receptors were used in assays to determine responses to odorants. While one receptor did not respond to any odorants tested, the second receptor responded to several odorants with a significant increase in intracellular IP$_3$ (Raming et al., 1993). This rise in second messenger levels in response to odor application indicated that the expressed olfactory receptors were acting as odorant receptors. Recently, a correlation between expression of a single receptor and sensitivity to a specific odorant was shown (Zhao et al., 1998). Zhao et al. (1998) used a recombinant adenovirus to increase the over expression of a single rat odorant receptor in olfactory receptor neurons. This increased receptor expression led to a higher sensitivity to a small subset of odorants (Zhao et al., 1998). These results provide indirect but compelling evidence that the putative odorant receptors are in fact functioning as odorant receptors. Additional ultrastructural work localized odorant receptor immunoreactivity to olfactory cilia, the site of ligand/receptor binding (Menco et al., 1997). Taken together, these studies provide growing evidence that the putative odorant receptors are acting as
specific receptors for odorants. Correlating specific ligand binding with these receptors will confirm their role in olfaction.

Buck and Axel (1991) found that the putative odorant receptors belonged to the G-protein coupled receptor superfamily. They deduced that the receptors contained hydrophobic domains that traverse the plasma membrane seven times, a characteristic of G protein coupled receptors. Despite this structural similarity, the authors found that the receptors exhibited significant sequence divergency, especially within transmembrane domains 3, 4, and 5. The authors postulated that this divergency could be used by the receptors to selectively bind to ligand and they proposed that the odorant ligand binding site resided within transmembrane domains 3, 4, or 5. The authors also hypothesized based on the sequence diversity they found that this receptor family would be very large, with 100 to 200 members and that these receptors would individually only be expressed in a small subset of neurons (Buck & Axel, 1991).

The same group later characterized olfactory receptors in catfish that shared considerable homology with the rat olfactory receptors, but were postulated to be much fewer in number (Ngai et al., 1993b). Other work had indicated in mammals that the gene family for odorant receptors consisted of approximately 1000 genes (Levy et al., 1991), while Ngai et al. (1993b) suggested that the catfish gene family consisted of only about 100 genes. Despite the differences between the two groups of receptors, Ngai et al.
(1993b) also found that the greatest area of diversity within subfamilies of odorant receptors in the catfish occurred between transmembrane domains 3 and 4, further supporting the hypothesis of Buck & Axel (1991) in rats that this area is the ligand binding site.

With the molecular characterization of the putative odorant receptors, interest in the cellular expression of these receptors intensified. It is believed that the expression pattern of odorant receptors will provide vital clues to understanding how the brain discerns so many odors. With this goal in mind, Ngai et al. (1993a) used in situ hybridization to determine the expression patterns of the putative odorant receptors in catfish. A specific receptor only hybridized to 0.5-2% of the olfactory neurons and that receptor expression seemed to be randomly distributed within the entire olfactory epithelium. Receptors from four distinct subfamilies were expressed in different and largely nonoverlapping subsets of neurons (Ngai et al., 1993a). In contrast, work in zebrafish determined that neurons projecting to the same glomerulus and presumably expressing the same receptor, were not randomly positioned in the olfactory epithelium but were evenly spaced from each other (Baier et al., 1994). The spatial organization of odorant receptor expression in zebrafish determined that the olfactory epithelium is divided into broad zones in which specific receptors are preferentially expressed. The expression domains found in fish were broadly overlapping compared to mammalian expression zones,
but the differences in receptor distribution within each zone were significant (Weth et al., 1996).

Based on in situ hybridization results which gave an indirect measure of the number of receptors found within a specific neuron, Ngai et al. (1993a) concluded that each neuron expresses only one or at most a few receptors, supporting the hypothesis that odors are discerned in the brain by the identity of the cell activated. In this model of odor perception, neurons with receptors that bind the same odorant all project their axons to a particular glomerulus within the olfactory bulb. In this case, even if individual receptors were randomly distributed throughout the olfactory epithelium, they would produce specific patterns in the olfactory bulb that could be used by the brain to discern which odor had been encountered (Ngai et al., 1993a).

In situ hybridizations in rat found that specific odorant receptor expression was restricted to one of several spatially segregated zones within the olfactory epithelium but that within a given zone, individual receptors were randomly expressed. The mammalian olfactory system may be so complex that the olfactory epithelium needed to be divided into discrete zones of receptor expression to allow for olfactory coding of the large number of possible odorants. Dividing the epithelium into zones of receptor expression provided another layer of organization to compensate for the complexity needed to be able to distinguish between thousands of odors (Vassar et al., 1993). Within other mammalian species, odorant receptors were also randomly expressed.
within one of several restricted zones (Strotmann et al., 1994a, 1994b, 1995),
leading to the hypothesis that this zonal patterning of odorant receptors may be
common to all mammals (Strotmann et al., 1995). These results correlate with
recent work that determined odorant receptors in zebrafish were expressed
within broad indistinct zones within the olfactory epithelium as well (Weth et al.,
1996).

Direct support for the hypothesis that odor quality in the olfactory bulb is
controlled by receptor activation in the peripheral neurons came from work in
genetically altered mice. In these mice, olfactory neurons that expressed
specific odorant receptors also expressed tau-lacZ which allowed visualization
of axon projections to the brain (Mombaerts et al., 1996). All the neurons
expressing a particular receptor projected to only two glomeruli within the
olfactory bulb. When the first receptor was replaced with the DNA for a second
receptor, the axons of the altered neurons projected to different glomeruli from
the first, but not to the same glomeruli as the wild type of the second receptor.
These results indicated that olfactory receptors themselves play some role in
determining which glomeruli their neurons synapse with, but that other factors
within the neuron must also control this process (Mombaerts et al., 1996).

The findings of Mombaerts et al. (1996) are supported by another study
in mice that substituted the coding sequence of one odorant receptor with
either nonsense mutations or the coding region of another odorant receptor
and then traced the projections to the olfactory bulb. The axonal projection of a
neuron with a nonsense mutation was not able to converge on specific glomeruli. They also found that substitutions with coding regions of different odorant receptors altered the neurons' ability to project to specific glomeruli. The authors concluded that odorant receptors play an important role in target selection but that there must be additional guidance receptors present (Wang et al., 1998). Further research defining stimulus coding in the olfactory bulb was done in zebrafish. Friedrich & Korsching (1997) found that stimulation by amino acids produced complex patterns of active glomeruli for different stimuli as well as for different concentrations of stimuli and that activity patterns were unique, even for odorants with similar structure (Friedrich & Korsching, 1997).

Mombaerts et al. (1996) and Wang et al. (1998) support the hypothesis that in addition to recognizing external olfactory stimuli, odorant receptors may belong to a larger chemosensory family of receptors that control numerous processes and can act as guidance controls. There is now growing evidence that odorant receptors and receptors very similar to odorant receptors are found outside the olfactory epithelium. These receptors are found in mammalian taste buds (Abe et al., 1993a; Abe et al., 1993b; Matsuoka et al., 1993; Thomas et al., 1996), testicular tissue (Vanderhaeghen et al., 1993; Thomas et al., 1996; Walensky et al., 1998), and spleen (Walensky et al., 1998). The same receptors were found in rat olfactory epithelium, taste epithelium and testicular tissue (Thomas et al., 1996). Odorant receptors have also been found in taste buds of channel catfish (see chapter two).
together, the combined results suggest that odorant receptors may function as general chemical recognition receptors with distinct ligand specificities determined by the cells in which they are expressed. Therefore, these receptors may function by directing developing cells' migration in the notochord (Nef & Nef; 1997), by detecting taste or olfactory stimuli (Buck & Axel, 1991; Abe et al., 1993a; Abe et al., 1993b; Ngai et al., 1993b; Matsuoka et al., 1993; Freitag et al., 1995; Issel-Tarver & Rine, 1996; Thomas et al., 1996), or by directing the movement of sperm cells (Vanderhaeghen et al., 1993; Thomas et al., 1996; Walensky et al., 1998) depending on where, as well as when, the receptors are expressed.

1.2 OTHER RECEPTORS EXPRESSED IN OLFACTORY NEURONS

In addition to multiple roles for odorant receptors, recent studies indicate that multiple types of receptors are present in olfactory neurons. Metabotropic glutamate receptors (mGluRs) were detected in the olfactory epithelium of Atlantic salmon (Pang et al., 1994), which corresponds to other work indicating that glutamate may act as the neurotransmitter at the olfactory nerve and mitral/tufted cell synapse (Berkowicz et al., 1994) as well as the neurotransmitter between the vomeronasal nerve and mitral cells (Dudley & Moss, 1995). Glutamate is one of the primary excitatory neurotransmitters found in the central nervous system and can stimulate either ionotropic or metabotropic receptors. Ionotropic receptors are ligand gated ion channels and are comprised of multimeric complexes of homologous subunits.
Metabotropic glutamate receptors are a multigene family of G-protein linked receptors that consist of 8 subtypes, which are divided into three groups based on amino acid homology and pharmacological profile. Members of this family of G-protein coupled receptors have a seven transmembrane domain region, but have no sequence homology to other known G-protein coupled receptors (Pin & Duvoisin, 1995).

Pang et al. (1994) characterized the presence of a mGluR in the olfactory epithelium of Atlantic salmon. Using fractions of olfactory plasma membrane, they characterized the binding of glutamate to this receptor and found that aspartate as well as known agonists and antagonists for mGluRs, effectively inhibited glutamate binding. Glutamate binding stimulated the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), indicating a G protein mediated activation of phospholipase C (PLC) (Pang et al., 1994), a known function of Group I mGluRs (Pin & Duvoisin, 1995). Finally, Northern blot analysis determined that cDNAs encoding mGluRs were able to hybridize to RNA from olfactory rosettes which indicated that a subtype of the mGluR family was present in the olfactory epithelium (Pang et al., 1994). Results of Pang et al. (1994) was substantiated by a study performed for this dissertation (see chapter 3) which determined that there are at least two subtypes of mGluRs (mGluR1 and mGluR3) present in the olfactory neurons of the channel catfish and that these receptors may modulate olfactory processes.
Recent work in other species has also determined that ionotropic glutamate receptors are present in the olfactory epithelium. Thukral et al. (1997) characterized the subunit composition of ionotropic glutamate receptors found within the olfactory epithelium of rats. Primarily, non-N-methyl-D-aspartate (non-NMDA) receptor subunits were found and immunocytochemical analysis indicated that these subunits were localized in the dendritic knobs of olfactory neurons as well as the olfactory neuron axon bundles that project to the olfactory bulb (Thukral et al., 1997). Electrophysiological evidence indicated the presence of at least two types of glutamate receptor found in the olfactory organ of the spiny lobster which were inhibited by NMDA and L-cysteine (Burgess & Derby, 1997). While Burgess and Derby (1997) were not able to determine what type of glutamate receptor they were characterizing, inhibition by NMDA strongly suggests that these receptors were ionotropic receptors.

While there is strong evidence indicating that multiple glutamate receptors are present in the olfactory epithelium, their role in olfaction is not well characterized. Evidence shows that glutamate may function as a neurotransmitter for olfactory neurons (Berkowicz et al., 1994) and that glutamate receptors in olfactory neurons may have a role as presynaptic receptors in this system, but little is understood about the presence of these receptors at the dendritic knobs of olfactory neurons (Thukral et al., 1997).
1.3 RECEPTOR REGULATION

Odorants stimulate vertebrate olfactory receptor neurons by binding with odorant receptors located in the dendritic cilia of the neurons. When ligand binds to these receptors, they activate G proteins (Bruch & Kalinoski, 1987) which result in the stimulation of either adenylate cyclase or phospholipase C (Breer et al., 1994; Bruch, 1996). These second messengers ultimately control the gating of ion channels involved in membrane depolarization which leads to the production of action potentials and synaptic transmission to the central nervous system. Second messengers are also involved in the termination of the olfactory signal by stimulating second messenger dependent protein kinases (Bruch et al., 1997a).

In the channel catfish, the primary second messenger pathway activated in response to odorant stimulation involves PLC (Bruch, 1996). Upon activation, PLC stimulates the breakdown of PIP$_2$ to IP$_3$ and diacylglycerol (DAG). IP$_3$ binds to the IP$_3$ receptor which is a ligand gated nonselective cation channel. This binding results in an increase of intracellular calcium which can in turn gate chloride channels and activate protein kinase C. When chloride channels open, chloride ions leave the cell and cause an enhancement in depolarization. Protein kinase C mediates many processes, including the heterologous desensitization of the odorant receptors.

Two known methods used by cells to desensitize 7 transmembrane domain (7TMD) receptors are homologous desensitization and heterologous
desensitization. After receptor activation, stimulated cellular processes cause the desensitization of the original, activated receptor. This process is called homologous desensitization and involves both G-protein coupled receptor kinases (GRKs) and arrestins. After ligand binding to the receptor, cytosolic GRKs translocate to the membrane and interact with $\beta_\gamma$ G-protein subunits which results in the anchoring of GRKs at the membrane. Subsequently, GRKs phosphorylate the activated receptor which causes some receptor inactivation and an increased binding affinity for arrestin. Once arrestin binds to the phosphorylated receptor, the receptor is completely inactive and is targeted for sequestration. After internalization, the receptors are either degraded or recycled to the plasma membrane (Chuang et al., 1996).

In the catfish olfactory system, at least some of the components used during homologous desensitization are present. In olfactory neurons, a G-protein coupled receptor kinase that was 92% identical to bovine $\beta$-adrenergic receptor kinase ($\beta$ARK2) has been characterized (Bruch et al., 1997a). Upon activation, $\beta$ARK2 has been shown to translocate and interact with G-protein $\beta_\gamma$ subunits at the cell membrane (Daaka et al., 1997). The $\beta_\gamma$ subunits present in olfactory receptor neurons have also been characterized in this system and it has been shown that the subunits $\beta 1$, $\beta 2$, $\gamma 2$, and $\gamma 3$ are present (Bruch et al., 1997b). All of the possible subunit combinations of these two subunits can interact with G-protein coupled receptor kinases (Simonds, 1994), and it has been shown that $\beta 1$ and $\beta 2$ can specifically interact with $\beta$ARK1 and $\beta$ARK2.
The G-protein $\beta y$ dimers may therefore interact with the GRK found in olfactory neurons to anchor it to the plasma membrane (Boekhoff et al., 1994).

Heterologous desensitization occurs when second messenger dependent kinases, protein kinase A (PKA) and protein kinase C (PKC), are activated due to a rise in cAMP or calcium and DAG levels within the cell. Upon activation, these protein kinases phosphorylate any 7-TMD receptors they contact, even if ligand is not present. While it is currently thought that phosphorylation of receptors occurs independently by these two desensitization processes, some studies have shown that second messenger dependent kinases can affect cellular expression of GRKs and arrestins. This allows these protein kinases to modulate the long term efficacy of homologous desensitization (Chuang et al., 1996). One study has also reported the direct phosphorylation of a GRK by PKC which greatly reduces GRK's ability to phosphorylate receptors (Pronin & Benovic, 1997). Therefore, PKC may have a direct role as well as an indirect role in receptor desensitization.

Odorant receptors contain consensus phosphorylation sites for PKC as well as PKA (Parmentier et al., 1994), and biochemical evidence has suggested that protein kinases can phosphorylate odorant receptors (Krieger et al., 1994). In the channel catfish, there are at least five subtypes of protein kinase C present in olfactory epithelium. These PKC subtypes consist of $\alpha$, $\beta$, $\delta$, $\varepsilon$, and $\theta$ subtypes (Bruch et al., 1997a) which belong to the conventional and novel subtype groups. Conventional PKC subtypes depend on calcium for
activation, while novel PKC subtypes are calcium independent (Liu, 1996).

Two PKC subtypes, β and δ, were found in olfactory receptor neurons (Bruch et al., 1997a). PKCβ is a conventional PKC and is dependent on the presence of calcium ions for activation, while PKCδ is a novel PKC and is calcium independent. This physiological difference between the PKC subtypes was exploited by Bruch et al. (1997a) to determine the subtype involved in odor induced phosphorylation. Bruch et al. (1997) performed *in vitro* phosphorylation assays on olfactory cilia from the channel catfish and found that odor stimulation caused a two fold increase in phosphorylation levels as measured by incorporation of radiolabeled phosphate from ATP. When a specific inhibitor of calcium-sensitive PKCs was added to the reaction mixture and the cilia were stimulated with odorant amino acids, phosphorylation was maintained at basal levels. These results indicated that the PKC activated in response to odor stimulation was sensitive to calcium. Based on the subtypes determined to be present in olfactory receptor neurons, it was concluded that the activated PKC was most likely PKCβ (Bruch et al., 1997a).

1.4 DISSERTATION SPECIFIC AIMS

This dissertation was designed to answer three specific questions: 1) Is there one or more than one odorant receptor gene expressed in an olfactory receptor neuron; 2) Are there G-protein coupled receptors present in olfactory receptor neurons in addition to odorant receptors; and 3) Can PKC phosphorylate and thus potentially regulate these G-protein coupled receptors?
To date, there is an unresolved issue between physiological data and molecular data concerning the number of receptor types present in olfactory receptor neurons. Electrophysiological studies (Ivanova & Caprio, 1993; Kashiwayanagi et al., 1996) have shown that individual neurons can be responsive to more than one odor stimuli. Biochemical evidence indicates that odorant receptors are relatively specific in their ligand binding (Bruch & Rulli, 1988). Taken together, this work provides strong indirect evidence that olfactory neurons contain multiple receptor types. However, molecular data indicated that individual olfactory neurons generally express one receptor type. This hypothesis was based on the observation that in situ hybridization of olfactory tissue showed that a specific receptor was only expressed in up to 2% of the total neuron population. Taken together with the estimated number of odorants that a particular species can detect, it was hypothesized that olfactory neurons do not express more than a few receptor types, with the general consensus being that there is probably really only one receptor present per cell (Ngai et al., 1993a).

It has been difficult to resolve the differences between molecular and electrophysiological data. Chapter two of this dissertation addresses this issue with the first known direct measure of odorant receptor expression in single olfactory neurons. Individual olfactory neurons were isolated and subjected to RT-PCR analysis to amplify odorant receptors. PCR products were analyzed by sequence analysis and genomic Southern blotting to determine if the
recovered products represented different receptors. This study determined that multiple receptors do exist in olfactory receptor neurons, despite the in situ hybridization studies but in agreement with electrophysiological data.

Chapter three addresses an issue that has not been well studied to date and concerns the presence of other types of G-protein coupled receptors in olfactory neurons. Most studies thus far have concentrated on characterizing the odorant receptors and have largely ignored the possibility that other receptors are present in olfactory neurons. In the Atlantic salmon, a metabotropic glutamate receptor is present in the olfactory epithelium (Pang et al., 1994) and additional studies have shown that ionotropic glutamate receptors are also present in the olfactory epithelium (Burgess & Derby, 1997; Thukral et al., 1997). Berkowicz et al. (1994) have shown that glutamate may act as a neurotransmitter between olfactory neurons and the olfactory bulb, indicating a possible role for glutamate receptors as presynaptic receptors.

Chapter three expands the characterization of mGluRs in the olfactory epithelium. This research determined whether these receptors were expressed in olfactory neurons and what receptor subtypes were present. It also established whether these receptors were expressed in the same olfactory neurons that expressed odorant receptors or if they were found in separate neurons. Immunocytochemical work localized the receptor protein within the neuron while electrophysiology work done in conjunction with Jim Parker in John Caprio's laboratory determined the response of these receptors to odor.
stimuli. It was shown that antagonists to mGluRs reduced the response of olfactory receptor neurons to glutamate to approximately half of normal. These data indicate that mGluRs do affect olfactory processes, at least during the detection of some amino acids odorants.

The final aim of this dissertation addresses the PKC phosphorylation of odorant receptors and metabotropic glutamate receptors found in olfactory neurons. In this study, purified PKCβ and PKCδ, two PKC subtypes previously shown to be present in olfactory receptor neurons (Bruch et al., 1997a), were used in phosphorylation assays with expressed proteins containing consensus phosphorylation sites found in odorant and glutamate receptors. The incorporation of radiolabeled phosphate from ATP was used to determine if the PKCs phosphorylated the consensus sites. For the substrate representing the mGluRs, both PKCβ and PKCδ were effective in phosphorylation. Results for the odorant receptor substrate indicated that the only consensus phosphorylation site present in these receptors was found within an extracellular domain. Assay results with this substrate found that PKCδ, but not PKCβ, phosphorylated this extracellular consensus site. Taken together with the in vitro study by Bruch et al. (1997a), it is likely that odor induced phosphorylation by PKCβ acts on other components of this pathway and not on the receptors themselves.
2.1 INTRODUCTION

An important factor in olfaction that has not been established is the number of odorant receptor genes expressed in individual olfactory neurons. Studies in rats, mice and catfish have concentrated on the whole olfactory tissue and have concluded that only a single odorant receptor is expressed within a neuron (Chess et al., 1994, Ngai et al., 1993a). In mice, Chess et al. (1994) used a "limiting dilution" approach in which they analyzed pools of cells ranging from 200 to 10,000 cells by RT-PCR and concluded that when the desired product was found in the 200 cell pools, the cell pool was likely to contain only a single cell expressing an odorant receptor PCR product (Chess et al., 1994). In catfish, these conclusions were based on in situ hybridization which seemed to indicate that a particular odorant receptor is only expressed in 1-2% of the olfactory neurons. Ngai et al. (1993a) theorized that due to the low expression of receptors within the olfactory tissue it is probable that there is only one odorant receptor per neuron. Similar conclusions based on in situ hybridization in rats and other mammals have also been made (Strotmann et al., 1994a; Strotmann et al., 1994b; Strotmann et al., 1995).

However, physiological studies in catfish indicate that olfactory receptor neurons can often respond to more than one odorant (Ivanova & Caprio, 1993),
while biochemical data show that odorant receptors are relatively specific in their ligand binding (Bruch & Rulli, 1988). These results conflict with the conclusions based on molecular biology data. In the present study, the RT-PCR method is used to directly determine the number of odorant receptor genes expressed within single olfactory neurons. This study shows that multiple odorant receptors are present in some olfactory receptor neurons of the channel catfish.

Odorant receptors have also been found in tissues other than the olfactory epithelium (Vanderhaeghen et al., 1993; Thomas et al., 1996; Nef & Nef, 1997). In rat, identical olfactory receptor like genes can be expressed in vallate taste bud, olfactory epithelium and testicular tissue (Thomas et al., 1996). Since the taste and olfactory systems of the channel catfish are both responsive to amino acids (Caprio, 1977), the possibility was investigated that receptors similar to the odorant receptors might be expressed in the taste system. Evidence is presented indicating that "odorant" receptors are also expressed in taste buds.

2.2 MATERIALS AND METHODS

Olfactory Neuron Isolation

Channel catfish (Ictalurus punctatus), 15-20 cm in length, were deeply anesthetized in MS-222 (1:5000) and sacrificed. Olfactory rosettes were removed and placed in fish Ringer's solution in a Sylgard coated 35 mm plastic dish. Olfactory neurons were obtained following the methodology of
Restrepo and Teeter (1990). Rosettes were bathed in calcium and magnesium free fish Ringer's solution and were subsequently dissociated in calcium and magnesium free fish Ringer's solution containing 30U/mL activated papain and 100µg/mL gentamicin. The tissue was bathed with this solution every 90 s for 15 min. Dissociation was terminated using fish Ringer's solution with 2mM CaCl₂, 10µg/mL leupeptin and 100µg/mL gentamicin. Olfactory receptor neurons were visualized by phase contrast microscopy and were identified by their morphological characteristics (Figure 2.1) (Bruch & Medler, 1996). Each cell was individually removed using suction attached to a 20µm bore siliconized (Sigmacote, Sigma Chemical Co., St. Louis, MO) microcapillary. Single neurons were placed into 100µL of lysis buffer (1% Igepal, 10mM Tris/HCl, pH 8, 140mM NaCl, 5mM KCl, 5mM DTT and 40 units RNAsin) and frozen on dry ice. Immediately prior to analysis, tubes were centrifuged at 12,000xg for 2 min to remove cellular and nuclear debris. The supernatant was removed to a fresh tube and used for RT-PCR.

RT-PCR Analysis of Odorant Receptor Expression in Olfactory Receptor Neurons

In order to determine the number of odorant receptors expressed within olfactory receptor neurons, individual neurons were analyzed. To further reduce the possibility of genomic contamination, the Dynabeads mRNA DIRECT kit (DYNAL, Oslo, Norway) was used to isolate poly (A)+ RNA from each neuron per the vendor's protocol. The resulting RNA on the Dynabeads
was eluted in sterile water and used for reverse transcription. Random primed reverse transcription was performed as previously described (Bruch & Medler, 1996). After termination of the reaction, the cDNA was used for PCR amplification as described by Ngai et al. (1993b) with the following modifications. Degenerate primers were made to the amino acid sequence of the transmembrane domains 3 and 7 and are shown in Table 1. All PCR analyses were initiated with Taq polymerase at 90°C. The cDNA was then amplified according to the following schedule: 94°C for 4 min, 38°C for 2 min, and 72°C for 1.5 min for 1 cycle, then 94°C for 1 min, 38°C for 2 min, and 72°C for 1.5 min for 39 cycles, followed by 72°C for 15 min with termination at 94°C for 15 min. The samples were slowly cooled to 4°C. Negative controls consisted of supporting cells that were treated identically as the neurons. Positive controls were run for each PCR reaction and consisted of a sequenced odorant receptor PCR product obtained from olfactory rosettes.

An aliquot of the PCR product was electrophoresed on a 2% agarose gel and the amplified DNA (approximately 550 bp) was excised and gel purified using the QIAEX II Gel Extraction kit (Qiagen, Chatsworth, CA). This product was ligated into pCR2.1 (Invitrogen, San Diego, CA), transformed into DH5α competent cells, and plated on LB plates with 50μg/mL ampicillin and 1mg/plate Xgal. Plasmids with inserts were identified by restriction digestion with EcoRI. Positive plasmids were grown in LB broth cultures and purified using columns from Qiagen (Chatsworth, CA) per the vendor's protocol.
Figure 2.1. Photomicrograph of dissociated cells from olfactory rosettes. The arrow in the middle of the picture indicates a typical olfactory receptor neuron, showing the bipolar morphology, round olfactory knob and axonal process. The arrow at the top of the picture indicates a supporting cell, showing the large round shape with a dense group of long cilia.
Products were sequenced on both strands using the Fidelity DNA Sequencing system (Oncor, Gaithersburg, MD) and were identified by comparison to the sequences in the data banks using the BLAST program (National Center for Biotechnology Information, Bethesda, MD). The Gene Runner program was used to obtain the predicted amino acid sequences (Hastings Software, Hastings, NY).

PCR primers previously used to amplify odorant receptors or G-coupled receptors in other systems were used to attempt amplification of odorant receptors in the catfish that may not have been amplified with the primers from Ngai et al. (1993b). Table 2.1 shows the primers used and references the source of the primers. In all cases, the PCR programs described in the original papers were used and controls were performed with rat tissues as appropriate. The identities of the amplified products obtained with each primer set were determined by DNA sequence analysis and are listed in the last column of Table 2.1.

Restriction Digestion with Frequent Cutting Endonucleases

Another method used to characterize the PCR products was to determine if more than one molecular species was amplified in the 550 bp band. Therefore, PCR products from individual olfactory receptor neurons were digested with the frequent cutting enzymes Hae III and Hinf I at 37°C for 1 hour. These products were separated on a 2% agarose gel and visualized with either ethidium bromide or Southern blot, depending on the band intensity.
Table 2.1. Primers used to amplify odorant receptors in the olfactory epithelium and olfactory neurons.

<table>
<thead>
<tr>
<th>PRIMERS USED</th>
<th>REFERENCE</th>
<th>PCR PRODUCTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-A-CGGAATTCGTATGGCITA(CT)GA(CT)(AC)G</td>
<td>Ngai et al., (1993b)</td>
<td>Catfish olfactory epithelium: catfish odorant receptors</td>
</tr>
<tr>
<td>5'-B-CGGAATTCGA(CT)(AC)GITA(CT)GTIGCAT(AC)TG</td>
<td>Freitag et al., (1995)</td>
<td>Catfish olfactory epithelium: catfish odorant receptors; β-adrenergic receptors</td>
</tr>
<tr>
<td>3'-C-GCTCTAGATA(AGT)AT(AG)AAIGG(AG)TTIA(AG)CAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-X2.2-TT(CT)AA(CT)(CT)T(AGCT)GC(CT)(GCT)T(AGCT)T(AGCT)GA</td>
<td>Freitag et al., (1995)</td>
<td>Catfish olfactory epithelium: catfish odorant receptors; (3-adrenergic receptors; metabotropic glutamate receptors</td>
</tr>
<tr>
<td>5'-X2.3-A(CT)(AC)CC(CT)ATGTA(CAT)TGTGCT(CT)T(CT)GT</td>
<td>Buck &amp; Axel (1991)</td>
<td>Catfish olfactory epithelium: catfish odorant receptors; β-adrenergic receptors; rat olfactory epithelium: metabotropic glutamate receptors</td>
</tr>
<tr>
<td>3'-X7.1-TA(AGT)AT(AG)A(CT)(AGT)GG(AG)TTIA(AGTAAT)CAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-A3-AA(T/C)(T/C)(T/A)ITT(T/C)(A/C)TIA(T/A)CICTGC(G/C)IGCIGA</td>
<td>Macrae et al., (1996)</td>
<td>Catfish olfactory epithelium: metabotropic glutamate receptors; catfish odorant receptors; β-adrenergic receptors; rat olfactory epithelium: metabotropic glutamate receptors</td>
</tr>
<tr>
<td>5'-A4-(C/A)GITT(G/C)(G/C)TTTGTGT(G/C)TIGCIGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'-B1-CTGI(CT)(G/T)(G/A)TTTATIA(A/T)(G/A)TTTTCATIA(A/T)(A/G)(C/G)TTTIGG(G/A)TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'-B5-AA(A/G)TCIGG(G/A)(C/G)(T/A)ICGI(G/G)AA(A/G)TAIA(T/C)IGG(G/A)TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-TM1-(C/T)TIGGCAA(C/T)(A/C/G)TIA(T/A)(C/T)IGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'-TM7-(C/G)(C/A/G)TAIA(C/G/T)(A/G)AAIGG(A/G)TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrae et al., (1996)</td>
<td></td>
<td>Catfish olfactory epithelium: metabotropic glutamate receptors; catfish odorant receptors</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
These bands were compared to molecular weight markers to determine their size and were summed to determine if there was one or more molecular species in the PCR product. These results were compared to a single known odorant receptor that was used as a positive control.

**Southern Blotting**

An aliquot of the receptor PCR products from isolated neurons was electrophoresed on a 2% agarose gel and transferred to a nylon membrane overnight with 10X SSC. The membrane was baked for one hour at 80°C and prehybridized for 2 hours at 42°C with buffer (50% formamide, 0.02% SDS, 0.1% N-Lauroylsarcosine, 2% blocking reagent, 5X SSC). Hybridization was performed overnight at 42°C with a digoxigenin-labeled cDNA probe (10ng/mL in buffer) prepared from an odorant receptor PCR product obtained from olfactory rosettes. The probe was gel purified and labeled with digoxigenin overnight with the Klenow enzyme (Boehringer Mannheim, Indianapolis, IN). After hybridization, the membranes were washed twice for 5 min each with 2X SSC/0.1% SDS at 42°C and twice for 15 min each with 0.5X SSC/0.1% SDS at 65°C. Hybrids were detected using alkaline phosphatase conjugated antibody to digoxigenin and chemiluminescent substrate CSPD (Boehringer Mannheim).

Individual PCR receptor product probes were also used to detect the odorant receptor genes present in the genome by Southern blot. Genomic DNA was obtained using DNAzol Reagent (Gibco, Grand Island, NY) by following the vendor's protocol. Four aliquots of 5μg each were digested with
either Eco RI, Eco RV, Bam HI or Hind III for 2 h at 37°C. These four digested genomic DNA products were separated on 0.3% agarose gel and were subjected to Southern blot as described above. After complete sequencing of the PCR products, individual probes were synthesized for each odorant receptor PCR product and used to hybridize to the genomic blot. If the banding patterns on the genomic DNA blot differed between the probes, the receptors were considered to have originated from different genes. Blots were stripped with boiling 0.1% SDS, cooled to room temperature and after prehybridization, were reprobed with additional odorant receptor PCR probes.

Odorant Receptor Expression in the Taste System

RT-PCR analysis was performed on RNA isolated with Trizol Reagent (Gibco, Grand Island, NY) per the reagent's protocol from olfactory and barbel epithelium of the channel catfish. Random primed reverse transcription was performed as previously described (Bruch & Medler, 1996). After termination of the reaction, the cDNA was used for PCR amplification as described earlier for the olfactory receptor neurons except for using the following primers: the 5' primer was primer 1-5'-ACCAAAGAAGCAATG-3' and the 3' primer was primer 2-5'-ATTATCCTATCATTGCATT733-3'. The primers 1 and 2 were designed to correspond to conserved regions within previously sequenced odorant receptor PCR products and contained no degeneracy. Primer 1 corresponded to the amino acids TKEAM and primer 2 to the amino acids NANDRI. Negative controls were run in which no reverse transcriptase was added to the sample.
The positive control consisted on a previously sequenced PCR product that had matched published odorant receptor sequence through the BLAST program. Bands of the appropriate size (400 bp) present in the PCR products of the catfish barbel were cloned and sequenced as described earlier for the olfactory receptor neuron PCR products.

Subsequent experiments utilized taste buds isolated from the barbel of the channel catfish and were graciously provided by Dr. Lidong Liu and Dr. Tim Gilbertson (Pennington Biomedical Research Center, Baton Rouge, LA). Poly(A)+ RNA samples were obtained from the taste bud and an olfactory receptor neuron and subjected to reverse transcription to obtain cDNA as previously described. This cDNA was used to amplify odorant receptors using primer 1 and primer 2 in the PCR program described earlier. The positive control used was an odorant receptor PCR product of known sequence, while the negative control was a sample with no reverse transcriptase. A band of the appropriate size (400 bp) was present in the positive control, the olfactory receptor neuron, and the taste bud lanes. Subsequent Southern blotting was performed to determine if the products hybridized to an odorant receptor probe.

2.3 RESULTS

RT-PCR Analysis of Odorant Receptor Expression in Olfactory Receptor Neurons

In order to amplify the odorant receptors present in individual olfactory receptor neurons, degenerate PCR primers were designed based on the amino
acid sequence of the transmembrane segments 3 and 7 of the odorant receptor family and were taken from Ngai et al. (1993b). Poly (A)+ RNA from the olfactory receptor neurons was individually isolated and used in the PCR program of Ngai et al. (1993b) with the modifications described earlier. PCR products of approximately 550 bp were analyzed from 19 olfactory receptor neurons. These products were subjected to Southern blotting, sequence analysis, and/or digestion with frequent cutting endonucleases. A positive control was run for each experiment using a PCR product of known sequence that was very similar to published odorant receptor sequences (Ngai et al., 1993b). Negative controls consisted of supporting cells that were identified morphologically and were presumed not to contain mRNA for olfactory receptors. In each of the negative controls, no bands were produced when the supporting cells were analyzed exactly as the olfactory receptor neurons.

Restriction Digestion with Frequent Cutting Endonucleases

PCR products from individual neurons digested with frequent cutting endonucleases were compared to a single product of known sequence (Figure 2.2). Hinf I did not cut the PCR products at all while Hae III cut it one time resulting in 2 bands of approximately 225 bp and 325 bp. The combined sizes of the 2 bands sum to the size of the uncut product (550 bp). These results suggested that a single species was present in the PCR product. Identical results were obtained with 8 additional olfactory neurons.
Figure 2.2. PCR products from single neurons digested with frequent cutting endonucleases. Molecular weight markers, shown in Lane 1, range from 100 bp to 1.5kb. Lanes 2 and 3 represent a known olfactory receptor PCR product that has been digested with Hae III (lane 2) and Hinf I (lane 3). Note that Hinf I did not cut the product (band size is 550 bp). Lanes 4 and 5 represent a PCR product obtained from a single olfactory receptor neuron digested with Hae III (lane 4) and Hinf I (lane 5). Lanes 6 and 7 represent a PCR product obtained from a second individual olfactory receptor neuron digested with Hae III (lane 6) and Hinf I (lane 7). Note that the same banding pattern is present for each product.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 2.3. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from 3 individual olfactory receptor neurons and indicated by A, B or C. The italicized sequence is published sequence of a putative odorant receptor (Ngai et al., 1993b). The products shared a high degree of sequence similarity to the published sequence and ranged from 93% to 95% identity with published sequence. Differences between the sequences are indicated with bold face type. Roman numerals indicate membrane spanning regions. (Lower panel) Southern blot analysis of 5µg genomic DNA digested with Bam HI (lane 1), EcoRI (lane 2), EcoRV (lane 3), or HindIII (lane 4). Odorant receptors from the sequences listed in the upper panel were used as probes and correspond to A, B, or C. Molecular weight standards ranging from 23kb to 0.5kb are shown on the far left of the blots.
Figure 2.4. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A through F. Products represent 4 different receptors and ranged from 90% to 95% sequence identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 23kb to 0.5kb are shown on the far left of the blots.
IV

TKEAMTLIIIVITWIFSISIAL-LVALITRLSCRSVIINSYFCDHPILILACNDKFI
ITWIFSITTIAL-LVALITRLSFCRSVIINNYFC-HGPIVILACNDKFI
TKEAMTLIIIVITWIFSINLPLHLLVALITRLSCRSVIINNYFCDHPVILACNDKFI
TKEAMTLIIIVITWIFSITTIAP-LVALITRLSFCRSVIINNYFCDHGPITVILACNDKFI
VITWIFSITTIAL-LVALITRLSFCRSVIINNYFCDHPVILACNDKFI
TKEAMTLIIIVITWIFSITTLAP-LVALITRLSFCRSVIINNYFCDHGPVILACNDKFI
VITWIFSITTIAL-LVALITRLSFCRSVIINNYFCDHPVILACNDKFI

V

NRVMAIGCFVVLDCVPLLIIVSYICIGIALMNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIACFVVLDCVPLLIIVSYICIGIALLNISHGLER-KAMKTCTSHLILVALFYL
NRVMAIACFVVLDCVPLLIIVSYICIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPLLIIVSYICIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPLLIIVSYICIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIACFVVLDCVPLLIIVSYICIGIALLNISHGLERRKAMKTCTSHLILVALFYL

VI

PFIGNITSLTSSINANDRIINSSLTQTIIIPPMNPFIY—published sequence
PFIGNITSLTSSINANDRIINSSLTQTIIIPPMNPFIY—A
PFIGNITSLTSSINANDRIINSSLTQTIIIPPMFNPIY—B
PFIGNITSLTSSINANDRIINSSLTQTIIIPPSRIPAHW—C
PFIGNITSLTSSINANDRIINSSLTQI—D
PFIGNITSLTSSINANDRIINSSLTQTIIIPPMFNPIY—E
PFIGNITSLTSSINANDRIINSSL—F

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 2.5. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A, B or C. Products represent 3 different receptors and ranged from 93% to 95% identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 23kb to 0.5kb are shown on the far left of the blots.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 2.6. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A or B. Products represent 2 different receptors and ranged from 92% to 95% identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 9kb to 0.5kb are shown on the far left of the blots.
Figure 2.7. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A, B or C. Products represent 2 different receptors and ranged from 76% to 95% identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 9kb to 0.5kb are shown on the far left of the blots.
Figure 2.8. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A or B. Products represent 2 different receptors and ranged from 91% to 95% identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 9kb to 0.5kb are shown on the far left of the blots.
Figure 2.9. (Upper panel) Sequence of PCR products of putative odorant receptors amplified from an individual olfactory receptor neuron and indicated by A, B or C. Products represent 3 different receptors and ranged from 91% to 95% identity with the published sequence. Refer to Figure 2.3 for explanation of figure labeling. Molecular weight standards ranging from 9kb to 0.5kb are shown on the far left of the blots.
Sequence Analysis of PCR products

Sequence analysis of 22 PCR products were obtained from 9 individual neurons and are shown in Figure 2.3 through Figure 2.9. For 3 individual neurons, only a single copy of the odorant receptor present was obtained per neuron. These sequences are shown in Figure 2.3 under a published sequence (Ngai et al., 1993b) in italics and are indicated by A, B, or C. The published sequence most closely matched all the odorant receptors obtained in this study and is shown in italics in Figures 2.3 through 2.9. Differences within the sequences are bolded. Percent identity between the published sequences and the sequences obtained in this study ranged from 76% to 95%. One sequence was 76% identical to the published sequence (see Figure 2.7) while the other 21 sequences were over 90% identical at the amino acid level.

Since all the amplified receptors had a high degree of sequence similarity, the possibility was investigated that there were other odorant receptors present in the neurons that were not being amplified by these particular primers. As a result, multiple experiments were performed using sets of primers that have been used to amplify odorant receptors in other animal models. One set of primers had previously been shown to amplify several types of G-protein coupled receptors in mammals (Macrae et al., 1996). All primers were utilized with their respective PCR programs and the G-protein coupled receptors that they amplified in the catfish olfactory epithelium are shown in Table 2.1. In the catfish, no other odorant receptors were amplified.
by any sets of the primers, including primers that amplified odorant receptors in the rat olfactory epithelium. While there may be other G-protein coupled receptors acting as odorant receptors within the olfactory epithelium, they were not detected with the sets of primers used from any of these studies.

Southern Blot of Genomic DNA

Due to the high degree of sequence identity, it was difficult to determine by sequence analysis alone whether multiple PCR products obtained from an individual neuron were copies of one receptor or represented multiple receptors. Therefore, each receptor was labeled with digoxigenin and hybridized to genomic DNA.

Genomic DNA was obtained from channel catfish brain and 5μg aliquots were digested with restriction enzymes. The digested products were separated by electrophoresis and analyzed by Southern blotting. The rationale used to determine receptor identity was based on the banding pattern produced by receptor probe hybridization. If the banding pattern between two receptor probes was identical, then it was concluded that those two receptors originated from the same gene and were multiple copies of the same receptor. Any differences in sequence between the two receptors with identical genomic blot patterns were assumed to be due to sequencing or PCR amplification errors.

Despite the high degree of sequence similarity between the receptors, multiple receptors were obtained from individual olfactory receptor neurons in most neurons studied. Figure 2.3 shows the genomic blots obtained for 3
receptors obtained from three different olfactory receptor neurons. Figure 2.4 represents 4 different receptors present within one neuron, while Figures 2.5 and 2.9 each show three different receptors in a single neuron. Figures 2.6, 2.7, and 2.8 each show two different receptors present in each individual neuron. Multiple copies of the same receptor were found in some neurons.

Odorant Receptor Expression in the Taste System

In the channel catfish, amino acids can serve as stimuli for both the olfactory and taste systems. Studies in other animal models have also shown that odorant receptors can be expressed in tissues other than the olfactory epithelium. Therefore, experiments were performed to determine if the "odorant" receptors were present in the catfish taste system as well. RT-PCR analysis was performed on RNA isolated from the barbel of a channel catfish. Fourteen PCR products from the catfish barbel were identified by sequence to correspond with catfish odorant receptors. The complete sequences of four PCR products were compared to the same published sequence shown in italics in Figures 2.3 through 2.9 and is also shown in italics in Figure 2.10. Products ranged from 87.5% to 95% identical to the published sequence.

Subsequent analysis of individual taste buds with RT-PCR produced comparable PCR products as compared to the positive control and an individual olfactory receptor neuron. A band of the appropriate size (400 bp) was present in the positive control, the olfactory receptor neuron, and the taste bud lanes. Subsequent Southern blotting with a known odorant receptor probe

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
| IV | DRVVAICFPLRNYIVTKEAMTLIIVITWIFSIISIALLVALITRLSFCSRVIINSYFC |
| V | DRYVAICFPLRNYAIVTKEAMTLIIVITWIFSTTIALLVALITRLSFCSRVIINSYFC |
|   | TKEAMTLIIVITWIFSIITIALLVALITRLSFCSRVIINNYFC |
|   | TKEAMTLIIVITWIFSIITIALLVALITRLSFCSRVIINNYFC |
|   | TKEAMTLIIVITWIFSIITIALLVALITRLSFCSRVIINNYFC |

Figure 2.10. Sequence of PCR products of putative odorant receptors amplified from catfish barbel epithelium. The italized sequence is published sequence of a putative odorant receptor (Ngai et al., 1993b). The products were found to share a high degree of sequence similarity to the published sequence (products ranged from 87.5% to 95% identical to published sequence). Differences between the sequences are indicated with bold face type. Roman numerals indicate membrane spanning regions.
Figure 2.11. Southern blot analysis of PCR products obtained from amplification of a known odorant receptor (lane 1), a taste bud (lane 3) and olfactory receptor neuron (lane 4). Lane 2 was the negative control. Products were hybridized with a known odorant receptor probe. Note that the banding pattern is the same for all three amplifications. Bands were approximately 400 bp in each of the lanes. Truncated PCR products are also present at approximately 300 bp.
confirmed that these bands contain odorant receptor products as the probe hybridized equally to the PCR product from all lanes (See Figure 2.11). This result was repeated in 2 additional taste buds with identical results (data not shown).

2.4 DISCUSSION

This study is the first to directly address the question of the number of odorant receptors expressed in olfactory receptor neurons. Degenerate PCR primers that had previously been shown to amplify odorant receptors in catfish olfactory epithelium (Ngai et al., 1993b) were used to amplify receptors in single neurons. These PCR products were analyzed by Southern blotting, sequence analysis and/or restriction digestion with frequent cutting endonucleases to determine the number of olfactory receptors expressed in single neurons. In every case, at least one odorant receptor was found. In most neurons analyzed by sequence analysis, 2 to 4 different odorant receptors were found. However, in three neurons subjected to sequence analysis, only one odorant receptor PCR product was found.

Restriction digestions with frequent cutting endonucleases indicated that only one odorant receptor PCR product was present in each neuron. PCR products were not cut with HindIII and were cut one time with Hae III to produce two bands of approximately 225 bp and 325 bp. Taken together, the sizes of these bands sum to the original band size of 550 bp, suggesting that only one species was present (Buck & Axel, 1991). However, subsequent sequence
analysis revealed that all the PCR receptor products were extremely similar to each other, with 76% to 95% of their sequences being identical. It is likely that due to the high degree of similarity between these receptors, the cut site within their sequences for Hae III was conserved among the receptors and that multiple receptors could not be detected with this methodology. More stringent methods were used, including sequence analysis of PCR products coupled with genomic Southern blots, to reveal the presence of multiple receptor genes in single neurons.

Due to the high degree of sequence similarity between the PCR products obtained in this study, a concern about the specificity of the primers became apparent. It was unclear if all the odorant receptors were being amplified from a given neuron, or whether these primers were only able to amplify a specific type of olfactory receptor. Other work in catfish using cDNA library screening had produced receptors that were much more varied in sequence (Ngai et al., 1993b). In order to address this concern, multiple primers, previously shown to amplify odorant receptors in a variety of animal models, were obtained (see Table 2.1). These primers were used in an attempt to amplify other types of olfactory receptors from the olfactory epithelium of the channel catfish. Table 2.1 lists the products obtained for each set of primers. For each set of primers, no unique olfactory receptor sequence was obtained. In the channel catfish, the primer sets either did not amplify odorant receptors or they amplified the same type of odorant receptor...
previously obtained. This was even true for a set of rat primers (Buck & Axel, 1991) that we used in the rat olfactory epithelium to obtain rat odorant receptors. It was not possible to obtain new types of odorant receptors in the channel catfish with these primers.

An interesting finding of this study was the extremely high similarity found among all the odorant receptor sequences. Twenty one of twenty two receptors from 9 different neurons were all over 90% identical to one published sequence (Ngai et al., 1993b). The one exception was still highly similar (76% identical). This finding differs from other studies (Buck & Axel, 1991; Ngai et al., 1993b; Freitag et al., 1995) which found multiple subfamilies of receptors present in the olfactory epithelium of their animal models. This difference may be due to one of several different factors. This study is the first to characterize odorant receptor gene expression in individual olfactory receptor neurons and the receptors amplified may be the predominant receptors expressed at the neuronal level. Another possibility is that the primers used in this study (Ngai et al., 1993b) were not able to amplify all the receptors present in the olfactory receptor neurons. For example, it has recently been shown that metabotropic glutamate receptors are present in olfactory receptor neurons and that these receptors may be acting, at least in part, as odorant receptors (Medler et al., 1998). The metabotropic glutamate receptors were not amplified by catfish odorant receptor primers even though they were coexpressed in the same cells with the odorant receptors. Finally, it is possible, though not likely, that the
specific neurons used in this study all happened to express the same receptor type and that if different neurons had been used, then different receptors would have been found.

Another interesting finding of this study was the presence of multiple receptors in single neurons. This result contrasts with the conclusions from previous studies (Ngai et al., 1993a; Chess et al., 1994; Strotmann et al., 1994a; Strotmann et al., 1994b; Strotmann et al., 1995) using molecular techniques suggesting that there is one receptor gene expressed per neuron. However, findings from this study do correlate with physiological and biochemical data showing that individual neurons can respond to multiple odorant stimuli (Ivanova & Caprio, 1993) and that odorant receptors are relatively specific in their ligand binding abilities (Bruch & Rulli, 1988). Multiple receptors were not found in all neurons tested, but this may be due to loss of mRNA during isolation procedures or other technical limitations. Alternatively, there may be a mixed population of neurons present in the olfactory epithelium in which some neurons express only one receptor gene while others express multiple receptor types. Based on morphological criteria, the cells studied in these experiments were mature, ciliated receptor neurons. Thus, the observed heterogeneity of odorant receptor expression is not related to neuronal maturation and turnover. Further work is needed to clarify these findings.

Lastly, this study determined the presence of odorant receptors transcripts in the taste buds of channel catfish. Although the ligands for
specific odorant receptors have not been identified, it is known that amino acids serve as stimuli for both taste and olfactory systems in the channel catfish (Caprio, 1977). Other studies have also shown that receptors very similar to odorant receptors can be found in mammalian taste buds (Abe et al., 1993a; Abe et al., 1993b; Matsuoka et al., 1993; Thomas et al., 1996), testicular tissues (Vanderhaeghen et al., 1993; Thomas et al., 1996; Walensky et al., 1998), and spleen, the site of lymphocyte storage (Walensky et al., 1998). One study indicated that the same receptors were found in the rat olfactory epithelium, the taste epithelium and testicular tissue (Thomas et al., 1996). In addition, Ngai et al. (1993b) amplified an odorant receptor using RT-PCR from catfish barbel but concluded that it was due to contaminating genomic DNA. Taken together, these findings suggested that odorant receptors may be expressed in the catfish taste system. Specific primers to a conserved region of the olfactory receptors were used to amplify products from catfish barbel epithelium that were highly similar to odorant receptors. Fourteen PCR products obtained from the barbel were identified as odorant receptors using a BLAST search (Genbank). The complete sequences of four receptor PCR products were at least 87% identical with previously published odorant receptor sequence (Ngai et al., 1993b) (see Figure 2.10). Further experiments using poly A+ RNA from taste buds and olfactory receptor neurons found identical PCR products between individual taste buds, single olfactory receptor neurons, and a known odorant receptor PCR product amplified as a
positive control. Southern blotting of the PCR products indicated that odorant receptors, or receptors similar enough to cross hybridize with odorant receptors, were present in the taste buds of the channel catfish (see Figure 2.11). This finding is consistent with the finding of odorant receptors in the barbel epithelium by sequence analysis. Clearly, these receptors are present in the barbel and are not the result of genomic DNA contamination as concluded by Ngai et al. (1993a).

This study shows for the first time in catfish that odorant receptors are present in the taste system as well as the olfactory receptor neurons. Due to the use of amino acids as stimuli by both systems in the catfish, it is not surprising that the same receptor types are found in both systems. Future work is needed to further characterize these receptors in the taste system so that their role in transducing taste stimuli can be determined. There is now growing evidence that odorant receptors may belong to a large family of receptors which have other physiological roles in addition to the recognition of odorants and taste stimuli. Multiple studies have now shown that receptors very similar to odorant receptors are present in a variety of tissues and during different life stages. These receptors may represent a family of receptors that function as general chemical recognition receptors with distinct ligand specificities determined by the cells in which they are expressed. Thus, these receptors may function by directing developing cells' migration in the notochord (Nef & Nef; 1997), by detecting taste or olfactory stimuli (Buck & Axel, 1991; Abe et
al., 1993a; Abe et al., 1993b; Ngai et al., 1993b; Matsuoka et al., 1993; Freitag et al., 1995; Issel-Tarver & Rine, 1996; Thomas et al., 1996), by guiding olfactory axons to glomeruli (Wang et al., 1998), or by directing the movement of sperm cells (Vanderhaeghen et al., 1993; Thomas et al., 1996; Walensky et al., 1998) or lymphocytes (Walensky et al., 1998) depending on where they are expressed. It is becoming apparent that the definition of these receptor types may need to be expanded to fully encompass their multiple, physiological roles before a complete understanding of their function is appreciated.
CHAPTER THREE. METABOTROPIC GLUTAMATE RECEPTOR EXPRESSION IN OLFACTORY RECEPTOR NEURONS FROM THE CHANNEL CATFISH, ICTALURUS PUNCTATUS*

3.1 INTRODUCTION

Metabotropic glutamate receptors (mGluR) are a multigene family of G-protein linked receptors that consist of 8 subtypes, which are divided into three groups based on amino acid homology and pharmacological profile. Group I receptors comprise mGluR1 and mGluR5 subtypes which stimulate phospholipase C activity. Group II includes mGluR2 and mGluR3 which are negatively coupled to adenylate cyclase. Group III receptors include the subtypes mGluR4, mGluR6, mGluR7, and mGluR8 which are also negatively coupled to adenylate cyclase. Members of this family of G-protein coupled receptors have a seven transmembrane domain region, but have no sequence homology to other known G-protein coupled receptors. These receptors are large (871 to 1199 amino acids) compared to other G-protein receptors and possess a large extracellular N-terminal domain (Schoepp, 1994). Splice variants have been found in some of these receptors and primarily affect the intracellular carboxyl tail after the seven transmembrane region of the protein. To date, splice variants have been found in the mGluR1, mGluR4, and mGluR5 subtypes (Pin & Duvoisin, 1995). There are several unique features of the mGluR family that distinguish it from the rest of the G-protein coupled receptor

*Reprinted by permission from John Wiley & Sons, Inc.
superfamily. One feature conserved across the family is an area in the extracellular domain and extracellular loops that is cysteine rich. Another unique feature of this receptor family is that the glutamate binding site is located in the extracellular domain instead of within the transmembrane domain (Conn & Pin, 1997). Finally, there is evidence from recent studies that at least some mGluRs exist in vivo as disulfide-linked dimers (Romano et al., 1996).

While the family of putative odorant G-protein coupled receptors have been characterized in several vertebrate species, their presence in the olfactory system does not exclude the possibility that other families of G-protein linked receptors may also have important functions in olfaction. In this study, the expression of metabotropic glutamate receptors was investigated in the olfactory rosette and in olfactory receptor neurons. This study characterized which subtypes were expressed in the olfactory rosette and where these receptors were localized within olfactory receptor neurons. Based on work in salmon (Pang et al., 1994) that found activation of a mGluR in the olfactory system led to PIP$_2$ hydrolysis and work in catfish that has shown the primary response to olfactory stimuli is the activation of PLC (Bruch, 1996), this study investigated the possible expression of mGluRs in the catfish olfactory system. Molecular cloning analysis of PCR products indicated that mGluR1 and mGluR3 subtypes were expressed in olfactory receptor neurons. Immunocytochemistry showed that both mGluR1 and mGluR3 subtypes were localized in the apical dendrites and some cilia of olfactory receptor neurons.
These receptors were molecularly characterized in single isolated olfactory receptor neurons and were found to be coexpressed with odorant receptors in the same cells. A novel observation of this study was that antagonists specific to mGluR1 and mGluR3 subtypes individually reduced olfactory receptor neural activity to the odorant, L-glutamate, to 55% of normal. Taken together, these results suggest that mGluRs may have a chemosensory function in the catfish olfactory system.

3.2 MATERIALS AND METHODS

RT-PCR Analysis of mGluR expression in Olfactory Tissue

Initial experiments were done on total RNA isolated from olfactory rosettes using Trizol Reagent (Gibco, Grand Island, NY) per the reagent's protocol. Random primed reverse transcription was performed as previously described (Bruch & Medler, 1996). After termination of the reaction, the cDNA was used for PCR amplification as described by Abe et al. (1992). Degenerate primers were made to the amino acid sequence of the transmembrane segments II and V of the mGluR family. The 5' primer was 5'-TCIAGICGIGA(A/G)CTITG(C/T)TA(C/T)AT and the 3' primer was 5'-TTICGIGT(C/T)TT(A/G)AA(A/C/G/T)GC(A/G)TA. All PCR analyses were initiated with Taq polymerase at 90°C. The cDNA was then amplified according to the following schedule: 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min for 50 cycles, followed by 72°C for 15 min with termination at 94°C for 15 min. The
samples were slowly cooled to 4°C. Negative controls were performed which lacked reverse transcriptase.

An aliquot of the PCR product was electrophoresed on a 2% agarose gel and the amplified DNA (450 bp) was excised and gel purified using the QIAEX II Gel Extraction kit (Qiagen, Chatsworth, CA). This product was ligated into pCR2.1 (Invitrogen, San Diego, CA), transformed into DH5α competent cells, and plated on LB plates with 50μg/mL ampicillin and 1mg/plate Xgal. Plasmids with inserts were identified by restriction digestion with EcoRI. Positive plasmids were grown in LB broth cultures and purified using columns from Qiagen (Chatsworth, CA) per the vendor’s protocol. Products were sequenced on both strands using the Fidelity DNA Sequencing system (Oncor, Gaithersburg, MD) and were identified by comparison to the sequences in the databanks using the BLAST program (National Center for Biotechnology Information, Bethesda, MD). The Gene Runner program was used to obtain the predicted amino acid sequences (Hastings Software, Hastings, NY).

Olfactory Neuron Isolation

Channel catfish (*Ictalurus punctatus*), 15-20 cm in length, were deeply anesthetized in MS-222 (1:5000) and sacrificed. Olfactory rosettes were removed and placed in fish Ringer’s solution in a Sylgard coated 35 mm plastic dish. Olfactory neurons were obtained following the methodology of Restrepo and Teeter (1990). Rosettes were bathed in calcium and magnesium free fish Ringer’s solution and were subsequently dissociated in calcium and
magnesium free fish Ringer's solution containing 30U/mL activated papain and
100µg/mL gentamicin. The tissue was bathed with this solution every 90 s for
15 min. Dissociation was terminated using fish Ringer's solution with 2mM
CaCl₂, 10µg/mL leupeptin and 100µg/mL gentamicin. Olfactory receptor
neurons were visualized by phase contrast microscopy and were identified by
their morphological characteristics (Bruch & Medler, 1996). Each cell was
individually removed using suction attached to a 20µm bore siliconized
(SigmaCote, Sigma Chemical Co., St. Louis, MO) microcapillary. Single
neurons were placed into 100µL of lysis buffer (1% Igepal, 10mM Tris/HCl, pH
8, 140mM NaCl, 5mM KCl, 5mM DTT and 40 units RNAsin) and frozen on dry
ice. Immediately prior to analysis, tubes were centrifuged at 12,000xg for 2 min
to remove cellular and nuclear debris. The supernatant was removed to a
fresh tube and used for RT-PCR.

**RT-PCR Analysis of mGluR Expression in Olfactory Receptor Neurons**

In order to determine which glutamate subtypes were found within
olfactory receptor neurons, individual neurons were analyzed. To further
reduce the possibility of genomic contamination, the Dynabeads mRNA
DIRECT kit (DYNAL, Oslo, Norway) was used to isolate poly (A)+ RNA from
each neuron per the vendor's protocol. The resulting RNA on the Dynabeads
was eluted in sterile water and used for reverse transcription. After termination
of the reverse transcription, the cDNA from each cell was divided equally for
PCR analysis. Negative controls lacking reverse transcriptase were also
performed with the analysis. Half of the cDNA from each cell was used to amplify glutamate receptors as described above and the other half of the cDNA was used to amplify odorant receptors (Ngai et al., 1993b) except that the 72°C extension step was shortened to 1.5 min and a total of 40 cycles was used. Products from 2 cells were subcloned and analyzed by DNA sequence. Products from subsequent analyses were characterized by Southern blot analysis using specific probes to identify glutamate and odorant receptor PCR products.

Southern Blotting

An aliquot of the receptor PCR products from isolated neurons were electrophoresed on a 2% agarose gel and transferred to a nylon membrane overnight with 10X SSC. The membrane was baked for 1 h at 80°C and prehybridized for 2 h at 42°C with buffer (50% formamide, 0.02% SDS, 0.1% N-Lauroylsarcosine, 2% blocking reagent, 5X SSC). Hybridization was performed overnight at 42°C with digoxigenin-labeled cDNA probes (10ng/mL in buffer) prepared from a glutamate receptor or odorant receptor PCR product obtained from olfactory rosettes. The probes were gel purified and labeled with digoxigenin overnight with the Klenow enzyme (Boehringer Mannheim, Indianapolis, IN).

After hybridization, the membranes were washed twice for 5 min each with 2X SSC/0.1% SDS at 42°C and twice for 15 min each with 0.5X SSC/0.1% SDS at 65°C. Hybrids were detected using alkaline phosphatase conjugated
antibody to digoxigenin and chemiluminescent substrate CSPD (Boehringer Mannheim).

mGluR Protein Localization

Metabotropic glutamate receptor protein localization was performed by immunocytochemistry. Olfactory rosettes were fixed by immersion in 4% paraformaldehyde in 0.1M PBS for 4 hours at 4°C. The tissue was then washed three times each for 15 min in PBS, quenched in 0.1M glycine for 30 min at room temperature and cryoprotected with serial changes of 10%, 20% and 25% sucrose in PBS for 1 h each at 4°C. The tissue was embedded in O.C.T. compound and twelve micron horizontal sections were cut on a cryostat and mounted on silanized slides for immunocytochemical staining. Slides were placed in 0.1% SDS in PBS for 20 min at room temperature and were then treated with 0.25% H₂O₂ in PBS for 30 min at room temperature. Nonspecific protein binding sites were blocked with diluted normal goat serum with 10μg/mL avidin (Sigma) at 37°C for 30 min. The primary antibodies were diluted with this normal serum and left on the slides overnight at 4°C. The next day the diluted secondary antibody was placed on the slides for 30 min at 37°C, followed by a 45 min incubation with newly made ABC reagent (Vector Laboratories, Burlingame, CA) at 37°C. All steps were followed by three 5 min washes with PBS except for the blocking step. Binding was visualized using the DAB peroxidase substrate kit (Vector Laboratories) as per the vendor's protocol for 5 min.
The primary antibody to mGluR1 was obtained from Upstate Biotechnology (Lake Placid, NY) and was produced to a 21 residue synthetic peptide (KPNVTYASVILDYKQSSSTL) corresponding to the C-terminus of mGluR1 with an additional lysine added at the N-terminus. The primary antibody to mGluR5 was also from Upstate Biotechnology (Lake Placid, NY) and was produced to a 21 residue synthetic peptide (KSSPKYDTLIRDYTNSSSSL) corresponding to the C-terminus of mGluR5 with an additional lysine added to the N-terminus. The mGluR group II antibody (Ohishi et al., 1994) was raised against a fusion protein containing a C-terminal portion of mGluR2 and had 80% amino acid sequence homology with the corresponding C-terminal residues 855-879 of mGluR3 (Ohishi et al., 1994). This antibody was shown to react with mGluR2 and mGluR3 and was graciously provided by R. Shigemoto (Kyoto University).

**In vivo Electrophysiology**

Each fish preparation was performed as previously described (Caprio et al., 1989; Caprio, 1995). A metal-filled glass micropipette was plated with platinum (ball diameter = 20 μm, impedance = 30KΩ). The electrode, r.c.-coupled (220 pF capacitor, 20 MΩ resistor) to one input grid of a high impedance probe, was placed against the surface of the epithelium of a single olfactory lamella. The other active input was grounded and connected to the reference electrode (hypodermic needle) embedded in the flank musculature. The multiunit activity was amplified (band pass 30-300 Hz), integrated (0.5 s...
rise time) and displayed on an oscilloscope and pen recorder. The response magnitude was measured in mm of the integrated phasic displacement from baseline and standardized to the response of the standard, either L-glutamate or L-methionine.

Charcoal-filtered artesian tap water (pH 8.5) was directed by polyethylene tubing to a glass capillary connected by polyethylene tubing to the olfactory organ which continuously bathed (10mL/min) the olfactory mucosa. Stimulus solutions were drawn hydrostatically from a disposable beaker and delivered through a second tube leading to the capillary. During odorant testing, the flow of water to the olfactory organ was instantly replaced with the test solution for 5 s by the activation of an electronic time switch. At the end of 5 s, the flow of water to the capillary was instantly resumed. This stimulus delivery system provided for the presentation of the odorant without a change in either pressure or temperature and with no dilution. The pH (8.5) of the gill irrigation water, the stimulus delivery water and test solutions was equilibrated to the pond water where the fish were obtained.

The antagonist used for subtype mGluR3 was the group II mGluR antagonist (α-Methyl-L-CCG l/(2S,3S,4S)-2-Methyl-2-(carboxycyclopropyl)glycine) (MCCG) (Tocris, Ballwin, MO). (S)-4-Carboxyphenylglycine ((S)-4CPG) was used as the antagonist for mGluR1 subtype (Tocris). Glutamate antagonists were diluted to 0.1 mM, pH balanced in charcoal filtered tap water to pH 8.5, and applied directly to the olfactory
epithelium for one min. Prior to the application of the antagonist, 1mM L-methionine and 1mM L-glutamate were tested as standards. Following standardization, one antagonist was presented continuously to the olfactory organ through the stimulus delivery system for one min followed immediately by the test of L-glutamate for 5 s. The antagonist was resumed for 10 s followed by a 5 s presentation of L-methionine and then a resumption of the water flow without antagonist for 4 min. Following this, the test scheme was repeated for the second antagonist.

3.3 RESULTS

Metabotropic Glutamate Receptors in Olfactory Tissue

Degenerate PCR primers were designed based on the amino acid sequence of the transmembrane segments II and V of the mGluR family and were taken from Abe et al. (1992). In order to determine which subtypes these primers would amplify in the catfish, total RNA from the olfactory epithelium was isolated and used in the PCR described previously (Abe et al., 1992). PCR products of approximately 450 bp were analyzed in which subtypes mGluR1 (16 clones) and mGluR3 (1 clone) were found. No band was present in the negative control. mGluR1 products had 75% nucleotide sequence similarity with rat and human mGluR1 sequences while the mGluR3 product was 69% similar to rat and human mGluR3 sequences. The carboxyl tail region of the mGluR1 was not amplified so it could not be determined which splice variants were present. At the amino acid level, there was 83% sequence
identity between the catfish mGluR1 and rat mGluR1 subtypes while there was 72% sequence identity between the mGluR3 subtypes (Figure 3.1). The high degree of sequence similarity suggested that both mGluR1 and mGluR3 subtypes were present in the olfactory epithelium.

**Metabotropic Glutamate Receptors in Olfactory Receptor Neurons**

Initial PCR experiments were performed in three individual neurons as previously described to determine which glutamate subtypes were present in the neurons. In one neuron 12 clones of mGluR1 were sequenced, another neuron had 7 mGluR1 and 3 mGluR3 clones identified, while in the third neuron, 1 mGluR3 and 4 mGluR1 subtypes were sequenced. Results indicated that both mGluR1 and mGluR3 subtypes were found in the olfactory receptor neurons and that both subtypes can be expressed within a single cell. Negative control lanes showed no bands present.

Subsequent experiments were performed to determine if glutamate receptors were coexpressed with the putative odorant receptors within individual neurons. After reverse transcription on individual neurons was complete, the cDNA was divided into two parts. One half of the cDNA was used to amplify the odorant receptors and the other half of the cDNA was used to amplify the glutamate receptors found in the same cell. In two neurons, the PCR products were subcloned and analyzed by sequence to determine which receptors were present. In one neuron, 3 odorant receptor clones and 4 mGluR1 clones were identified. In the second neuron, 6 odorant receptor
Figure 3.1. Sequence of mGluR PCR products amplified from olfactory epithelium. Two products were found that shared a high degree of sequence similarity to the rat mGluRs (mGluR1 was 83% identical to rat and mGluR3 was 72% identical to rat). Differences between the sequences are indicated with bold face type. Roman numerals indicate membrane spanning regions.
clones, 6 mGluR1 clones and 1 mGluR3 clone were identified (Figure 3.2). Subsequent PCR products obtained from 10 additional individual neurons were analyzed by Southern blotting to determine if the PCR products were odorant or glutamate receptors (Figure 3.3). Southern blotting was performed under stringent conditions using dioxigenin-labeled odorant receptor or glutamate receptor probes. Previous experiments indicated that the probes did not cross hybridize to each other (data not shown). The PCR products from each neuron hybridized to their respective probes indicating that both the putative odorant receptors and glutamate receptors were amplified from a single neuron in all neurons tested.

**mGluR Protein Localization**

In order to localize the area of the neuron that contained the glutamate receptor protein, immunocytochemistry was performed using antibodies specific to the mGluR1 and Group II subtypes. Specific labeling was observed in the dendritic knobs and some cilia in the chemosensory region for both subtype 1 and 3 (Figure 3.4, A&B). Staining for both subtypes was approximately equal although the staining was less intense in the cilia. Nonspecific staining was minimized by the pretreatment of the slides with both hydrogen peroxide to remove endogenous peroxidase activity and avidin to bind to endogenous biotin present. In the absence of primary antibody, no specific staining was evident (Figure 3.4C). In the presence of an antibody specific for mGluR5, specific staining was also absent (Figure 3.4D).
Figure 3.2. Sequence of PCR products of putative odorant receptors amplified from olfactory receptor neurons that were coexpressed with the mGluRs. The italicized sequence is published sequence of a putative odorant receptor (Ngai et al., 1993b). The next six sequences were from a single olfactory receptor neuron and were coexpressed with both mGluR1 and mGluR3 subtypes. The last three sequences are products expressed in another olfactory receptor neuron that also expressed mGluR1. Roman numerals indicate membrane spanning regions. Differences found between the sequences are indicated with bold face type. All sequences were >90% identical with the published putative odorant receptor sequence.
IV

TKEAMTIIIVITWIFSIITIAL - LVALITRLSFCRSVIINSYFCDHGPILILACNDKFI
ITWIFSITTIAL - LVALITRLSFCRSVIINNYFC-HGPIVILACNDKFI
TKEAMTIIIVITWIFSINITLPLLHLVALITRLSFCRSVIINNYFCDHGPILILACNDKFI
TKEAMTIIIVITWIFSITTIAP - LVALITRLSFCRSVIINNYFCDHGPITVLACNDKFI
VITWIFSITTIAL - LVALITRLSFCRSVIINNYFCDHGPILILACNDKFI
TKEAMTIIIVITWIFSITTIAL - LVALITRLSFCRSVIINNYFCDHGPILILACNDKFI
VITWIFSITTIAL - LVALITRLSFCRSVIINNYFCDHGPILILACNDKFI

V

NRVMAIGCFVVLDCVPFLIIIVSYCIGIALMNIISHGLERRKAMKTCTSHLILVALFYL
NRVMAIACFVVLDCVPFLIIIVSYCIGIALLNIISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL
NRVMAIGCFVVLDCVPFLIIIVSYCIGIALLNISHGLERRKAMKTCTSHLILVALFYL

VI

PFFIGTNITSLTSSINANDRIIINSTLTQIIPPMNPFLYY - published sequence
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-1-2
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-1-16
PFFIGTNITSLTSSINANDRIINSLTQIIPPSNIPAY-1-4
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-1-13
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-1-38
PFFIGTNITSLTSSINANDRIINSL-1-24
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-2-17
PFFIGTNI-2-25
PFFIGTNI-2-25
PFFIGTNI-2-25
PFFIGTNI-2-25
PFFIGTNITSLTSSINANDRIINSLTQIIPPMNPFLYY-2-41

768

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 3.3. Southern blots of PCR products obtained from individual olfactory receptor neurons. The left panel represents metabotropic glutamate receptor PCR products amplified from half the cDNA of a single neuron and hybridized with a known glutamate receptor probe. Lane 1 is a positive control, lane 2 is a negative control, lane 3 and lane 4 each represent PCR products from single olfactory receptor neurons. Truncated PCR products are present in both sample lanes as well as the positive control lane. The positive control was a cloned PCR product that had been sequenced and had previously been shown to be a mGluR subtype. Film was exposed to the chemiluminescent blot for 5 minutes.

The right panel represents putative odorant receptor PCR products amplified from the same neurons as the mGluRs and hybridized with a known odorant receptor probe. Lane 1 is a positive control, lane 2 is a negative control, lane 3 and lane 4 each represent PCR products from single olfactory receptor neurons. Film was exposed to the chemiluminescent blot for 3 minutes to develop the positive control and for 15 minutes to develop lanes 2, 3 and 4. The positive control was a cloned PCR product that had been sequenced and shown to be a putative odorant receptor.
Figure 3.4. Micrographs of the localization of mGluRs by immunocytochemistry. Panel A shows the staining of the chemosensory region of the olfactory epithelium in the channel catfish with mGluR1 antibody. Panel B shows staining of the olfactory epithelium with mGluR2/3 antibody. Staining is evident in the dendritic knobs and cilia in both A and B. Panel C is the negative control in which no primary antibody was used. Panel D shows epithelium stained with mGluR5 antibody. (600X)
In vivo Electrophysiology

Multi-unit recordings within the olfactory rosette of channel catfish were performed to determine if mGluRs are involved in olfactory responses. In this technique, electrical responses are recorded from the surface of several olfactory neurons simultaneously. Antagonists specific to certain mGluR subtypes were used. S-4CPG is a known antagonist for mGluR1 subtypes (Pin & Duvoisin, 1995) while in the rat spinal cord, MCCG has been shown to act as an antagonist to Group II mGluR subtypes (Jane et al., 1994). Recent studies have shown that MCCG is a more potent antagonist for mGluR3 as compared to mGluR2 (McCool et al., 1996). Each antagonist was individually applied to the olfactory epithelium and its effect on the L-glutamate induced response was measured. These experiments were duplicated with a L-methionine standard as a control for non-specific effects of the antagonists. Responses to glutamate in the presence of antagonist were first compared to the standard L-glutamate response in the absence of antagonist and were analyzed by Wilcoxon Signed Rank test. Both antagonists significantly reduced the glutamate response (p=0.02 for both) and also significantly reduced the methionine response (p=0.02 for both) as compared to the methionine standard. Representative recordings are shown in Figure 3.5.

Glutamate and methionine responses with antagonist were then expressed as percent reductions of the standard response for each stimulus for both antagonists. Methionine was reduced to 73% of the standard response.
Figure 3.5. Effect of the glutamate antagonists, S-4CPG (left) and MCCG (right), on integrated olfactory receptor responses to L-glutamate (top) and L-methionine (bottom). C, water control; A, L-glutamate standard; B, L-glutamate with S-4CPG; D, L-glutamate with MCCG; A', L-methionine standard; B', L-methionine with S-4CPG; D', L-methionine with MCCG.
Figure 3.6. Graphical representation of the calculated peak areas for glutamate and methionine responses in the presence of mGluR antagonists. The area for each peak reduced by antagonist was converted into a percent reduction of the control peak (n=7). Values were determined for both glutamate and methionine in the presence of each antagonist and median values were calculated. Error bars represent the interquartile range for each data set. Lane 1 represents the median reduction caused by MCCG on L-glutamate response while lane 2 represents the median reduction caused by MCCG on L-methionine response. Lane 3 represents the median reduction caused by S-4CPG on L-glutamate response while lane 4 represents the median reduction caused by S-4CPG on L-methionine response.
by S-4CPG and to 76% of standard response by MCCG while the glutamate response was reduced to 54% of standard by S-4CPG and 56% by MCCG (Figure 3.6). Wilcoxon Signed Rank tests for each antagonist were performed between glutamate and methionine. Significant differences between odorant receptor responses to glutamate and to methionine were found for each antagonist (p=0.02 for both).

3.4 DISCUSSION

Degenerate PCR primers were designed based on the amino acid sequence of the transmembrane segments II and V of the mGluR family (Abe et al., 1992). PCR products of approximately 450 bp were obtained from olfactory rosettes that corresponded to subtypes mGluR1 (16 clones) and mGluR3 (1 clone). The carboxyl tail region of the mGluR1 was not amplified so it could not be determined which splice variants were present. At the amino acid level, there was 83% sequence identity between the catfish mGluR1 and rat mGluR1 subtypes while there was 72% sequence identity between the mGluR3 subtypes. The high degree of sequence similarity suggested that both mGluR1 and mGluR3 subtypes were present in the olfactory epithelium. PCR products of the appropriate size were not obtained from negative controls lacking reverse transcriptase, suggesting that mGluR PCR products were not amplified from genomic DNA.

In order to characterize the cell types expressing the mGluR subtypes in the olfactory epithelium, individual olfactory receptor neurons were analyzed.
RT-PCR analysis of individual cells indicated that both subtypes mGluR1 and mGluR3 were present in olfactory receptor neurons and were coexpressed in the same neurons. In each RT-PCR reaction done on individual cells, precautions were taken to minimize genomic DNA contamination. First, prior to the analysis, each tube containing the lysed neuron was centrifuged to remove the nucleus. Second, poly-A+ RNA was used as template in RT-PCR. Negative controls lacking reverse transcriptase did not produce PCR products, indicating a lack of genomic DNA contamination.

Another aspect of this study was the novel determination that the mGluRs were co-expressed in the same olfactory receptor neurons with the putative odorant receptors. Individual neurons were isolated and underwent reverse transcription. The cDNA was then split into two tubes and PCR analysis was performed on each. One tube was used to amplify the putative odorant receptors while the other tube was used to amplify mGluRs. This analysis indicated that mGluRs and odorant receptors were expressed in the same cells. Furthermore, both mGluR1 and mGluR3 were expressed in the same cells as the odorant receptors. These results were obtained by sequence analysis of PCR products from two individual olfactory receptor neurons. Analysis of ten additional neurons by Southern blot analysis indicated that both receptor types were expressed in all cells tested. To our knowledge, this is the first reported study that shows at the cellular level,
mGluRs and odorant receptors are present in the same olfactory receptor neurons.

Two additional PCR products similar to mGluR1 were also isolated that did not have the same sequence or same genomic Southern blot pattern as the identified subtype (data not shown). Additionally, the sequence of these products was unlike any other known mGluR sequence. Initially, it was predicted that due to the high degree of identity between mGluR1 and mGluR5, one of the novel products was actually the mGluR5 subtype. Immunocytochemical data did not support this hypothesis. It is possible that these products are novel mGluR subtypes. There is evidence in other systems that novel mGluR subtypes are present that have not yet been characterized (Conn & Pin, 1997).

Pang et al. (1994) reported finding a mGluR subtype in the olfactory rosette of the Atlantic salmon, showing the presence of these receptors in the olfactory tissue of fish. The present study definitively showed that these receptors were expressed in olfactory neurons. Further, while Pang et al. (1994) did not classify which subtypes were present in the epithelium, the present study was able to identify by sequence that both mGluR1 and mGluR3 were present in the olfactory receptor neurons. This is in contrast to previous work in the rat taste system in which the mGluR4 subtype was identified (Chaudhari et al., 1996).
Pang et al. (1994) linked the presence of a glutamate receptor to IP₃ formation using an enzyme assay. The present study reinforced these results from Pang et al. (1994) by the identification of mGluR1 which is known to be positively coupled to PLC (Conn & Pin, 1997). Further, Northern blot analysis performed by Pang et al. (1994) found that probes to mGluR1 and mGluR4 hybridized to total RNA from the olfactory rosette. The DNA probes used in the present study on catfish were not able to distinguish subtypes due to cross hybridization (data not shown). Cross reactivity of the Northern probes was not discussed by Pang et al. (1994) and it is possible that the probes used were not specific to receptor subtype. Analyzing PCR products by sequence allowed the present study to determine that mGluR1 and mGluR3 subtypes were present in the catfish olfactory epithelium. No other known subtypes were characterized. Finally, the present study found that the mGluR subtypes were coexpressed in the same individual olfactory receptor neurons and furthermore, that these receptors were also coexpressed with the odorant receptors.

After characterizing the expression of mGluR1 and mGluR3 in olfactory receptor neurons, the neuronal localization of the mGluR proteins was determined. Immunocytochemical analysis of sectioned olfactory epithelium suggested that these receptors are expressed in the apical dendrites and possibly cilia of olfactory receptor neurons. Examination of the stained sections revealed consistent staining of almost all the dendrites and some of the cilia found within the chemosensory region of the epithelium.
Antibody specificity for each subtype was tested by running negative controls. Non-specific staining was reduced by pretreatment of slides with hydrogen peroxide to reduce peroxidase activity. Preadsorption of avidin to the slides reduced non-specific binding to biotin. Negative controls indicated that staining was not due to nonspecific effects. Further, slides stained with mGluR5 antibody showed no specific immunoreactivity. These results further confirm antibody specificity because mGluR5 is approximately 45% identical to group II receptors and 65% identical to mGluR1 in the rat (Pin & Duvoisin, 1995).

Pharmacological studies were performed to determine if these receptors affect odorant signaling. S-4CPG is a specific antagonist to mGluR1 (Conn & Pin, 1997; Pin & Duvoisin, 1995; Batchelor et al., 1997), while MCCG has been shown as a selective antagonist for Group II subtypes (Conn & Pin, 1997; Jane et al., 1994). Responses to known odorants were first recorded in the absence of antagonists to be used as standards and were then recorded after antagonists had been applied to the epithelium through the bathing medium. Each antagonist significantly reduced the signal caused by odorant application. The response to glutamate was reduced to 54% of standard by S-4CPG and reduced to 56% of standard by MCCG while the response to methionine was reduced to 73% of standard by S-4CPG and to 76% of standard by MCCG. The intensity of the antagonist effect was significantly greater for the glutamate response as compared to the methionine response. The methionine response
may have been affected by the glutamate antagonists because these antagonists are glycine derivatives. Glycine has been shown to cross react to methionine receptors by about 30% (Caprio & Byrd, 1984) and it is possible that these antagonists may be able to compete with methionine for their binding sites. Another possible reason that the methionine response was affected by these antagonists is that these mGluRs are functioning in a general modulatory role within olfaction. Further studies are needed to test this hypothesis.

These data further support a function for mGluRs in olfaction and show for the first time that antagonists to these subtypes reduce the odorant response. The remainder of the glutamate response may be due to other mGluRs present in the tissue. Two PCR products similar to mGluR1 were identified that did not have the same sequence as a known mGluR subtype. These novel products may represent additional mGluR subtypes that have not yet been characterized but may be responsible for some of the glutamate odorant response. The glutamate response may also be partially due to ionotropic glutamate receptors present in the olfactory epithelium. This study did not address the presence of these receptors in the olfactory tissue; however, a study in rat (Thukral et al., 1997) found ionotropic glutamate receptor subunits in the olfactory epithelium and showed that the proteins were primarily localized in the dendritic knobs of the olfactory receptor neurons. Finally, there may be an odorant receptor belonging to another G-protein coupled family of receptors that responds to glutamate. Any of these receptors
may be contributing to the glutamate response and prevent complete reduction of the olfactory response by mGluR antagonists.

This study presents the first evidence linking mGluRs to olfaction in the channel catfish. PCR analysis of individual olfactory receptor neurons found that mGluR1 and mGluR3 subtypes are expressed in the neurons while immunocytochemistry showed the proteins were localized in the dendritic knobs and some cilia, the site of olfactory transduction. Electrophysiological experiments found that antagonists to these mGluR subtypes inhibited the response to the odorant glutamate significantly greater than their effects on methionine. The combined data are consistent with the conclusion that mGluRs are localized in the cilia and may, at least partially, act as glutamate odorant receptors. This is in agreement with another study showing that mGluRs can act as sensory receptors. Chaudhari et al. (1996) found that in the rat taste system, mGluR4 seems to act in part as the taste receptor for monosodium glutamate (MSG). However, the widespread distribution of the mGluRs in the catfish epithelium does not agree with previous work indicating that individual odorant receptors are not highly expressed throughout the epithelium (Ngai et al., 1993a). While it is possible that mGluRs may be acting as glutamate odorant receptors, their high density in the epithelium suggests that these receptors may have additional, perhaps modulatory, functions in olfactory receptor neurons. However, there are no data currently available showing that glutamate modulates olfactory responses to other odorants.
3.5 END NOTE

CHAPTER FOUR. PHOSPHORYLATION BY PROTEIN KINASE C OF ODORANT RECEPTORS AND METABOTROPIC GLUTAMATE RECEPTORS

4.1 INTRODUCTION

Transducing a signal from the environment to internal cellular components is necessary for a cell to appropriately function in its environment. While there are several types of cell surface receptors that interact with extracellular stimuli, the seven transmembrane domain (7TMD) receptors seem to comprise the largest group, with over 1000 gene members. These 7TMD receptors function by coupling to heterotrimeric G proteins, which increase or decrease second messenger levels within cells. When ligand binds, 7TMD receptors undergo a conformational change which results in the activation of G-proteins. This signal is maintained until the receptors are rendered inactive during the process of desensitization.

One group of receptors that is thought to comprise most of the members of the 7TMD receptor superfamily are the putative odorant receptors. In rat, it is estimated that there are 1000 genes in the olfactory receptor subfamily (Buck & Axel, 1991) while in catfish, there are thought to be about 100 receptors (Ngai et al., 1993b). These receptors are believed to transduce odor stimuli from the external environment into the cell through G-protein activated second messenger production. These second messengers modulate nonselective cation channels and cause membrane depolarization.
Attenuation of an odor signal is needed to reset the cell’s sensitivity to future stimuli as well as terminating the signal being sent to the olfactory bulb.

Putative odorant receptors contain consensus phosphorylation sites for PKC as well as protein kinase A (PKA) (Parmentier et al., 1994) and biochemical evidence supports the hypothesis that protein kinases phosphorylate odorant receptors (Krieger et al., 1994). Work done in catfish has shown that at least five subtypes of protein kinase C as well as βARK2 are present in olfactory epithelium. These PKC subtypes consist of α, β, δ, ε, and θ subtypes, two of which, β and δ, have been found in the olfactory receptor neurons, the site of odor transduction (Bruch et al., 1997a). PKCβ is dependent on the presence of calcium ions for activation, while PKCδ is not. This physiological difference between the PKC subtypes was utilized to determine which subtype was involved in odor induced phosphorylation.

Phosphorylation assays performed on olfactory cilia from the channel catfish found that stimulation by odors resulted in a two fold increase in phosphorylation. In the presence of a specific inhibitor of calcium-sensitive PKCs, phosphorylation was reduced to basal levels after cilia were stimulated by odors. These results indicated that the PKC activated in response to odor stimulation was sensitive to calcium and was most likely PKCβ (Bruch et al., 1997a).

Another 7TMD receptor family that has recently been characterized in olfactory receptor neurons of the channel catfish is the metabotropic glutamate
receptor (mGluR) family. Two subtypes were found that were closely related to mGluR1 and mGluR3. These receptor subtypes were coexpressed with each other and with odorant receptors in individual olfactory receptor neurons. Antagonists to these receptor subtypes also significantly inhibited odorant responses in vivo, suggesting a possible role for these receptors in olfaction (Medler et al., 1998). mGluRs also undergo phosphorylation by protein kinases and contain consensus sites for PKC. To date, there is also no evidence that group I mGluRs undergo homologous desensitization (Gereau & Heinemann, 1998). That would make the role of PKC phosphorylation even more pivotal for these receptors.

One study has shown that amino acids C694-T695 found in the second intracellular domain of Group I mGluRs are important residues for interaction of the receptor with $G_\alpha$ (Francesconi & Duvoisin, 1998). These amino acids are also integral members of a potential phosphorylation site for PKC (Pinna & Ruzzene, 1996). Taken together, these findings suggest that important sites of interactions for the receptor with ligand and/or effectors may also correlate with phosphorylation sites for protein kinases. One way for protein kinases to alter receptor mediated processes would be through interference at the point of interaction with effectors.

In this study, protein segments from odorant receptors and mGluRs that contain consensus phosphorylation sites for PKC were generated. For both odorant and glutamate receptors, the serine/threonine residues in the
consensus PKC phosphorylation sites were mutated to alanine. The mutated and wild type proteins for both odorant and glutamate receptors were used in phosphorylation assays as substrates for the PKC subtypes PKCβ and PKCδ previously shown to be expressed in olfactory receptor neurons (Bruch et al., 1997a). These assays determined which PKC subtype, if any, could selectively interact with the potential phosphorylation sites tested.

4.2 MATERIALS AND METHODS

Protein Expression in Xpress System

PCR products for metabotropic glutamate receptors (approximately 400 bp) and odorant receptors (approximately 550 bp) obtained from catfish olfactory receptor neurons were used for protein expression in the Xpress System (Invitrogen, San Diego, CA). DNA fragments obtained from PCR analyses were subcloned into the appropriate pTrcHis vectors for expression. To subclone the odorant receptor PCR product in the right orientation, the pTrcHis expression vector was treated with alkaline phosphatase (Stratagene, La Jolla, CA) at 37°C for 30 min. Alkaline phosphatase was then heat inactivated for 15 min at 75°C and the insert was ligated into the vector at 22°C overnight. Glutamate PCR products were ligated into the expression vector without treatment of alkaline phosphatase. Ligated products were transformed into DH5α competent cells and plated on LB plates with 50μg/mL ampicillin. Plasmids with inserts were identified by restriction digestion with EcoRI. Positive plasmids were grown in LB broth cultures and purified using columns.
from Qiagen (Chatsworth, CA) per the vendor's protocol. Products were sequenced using the Fidelity DNA Sequencing system (Oncor, Gaithersburg, MD) to verify that the insert was in the right orientation and in frame with the expression vector.

LB broth culture aliquots were then used in a pilot expression study to determine expression levels and solubility of the protein. Cell growth was monitored at 600nm until the OD was approximately 0.6. Protein expression was induced with isopropylthio-β-D-galactoside (IPTG) (final concentration 1 mM) and hourly aliquots were taken for 3 h to monitor the expression. For each aliquot, cells were pelleted and the supernatant was removed. Pellets were resuspended in 50μL of 20mM phosphate buffer, pH 7.3, and incubated with 50μg of lysozyme for 5 min. Samples were boiled for 90 s and centrifuged for 10 min to separate the pellet from the supernatant. Supernatants were transferred to a fresh tube and mixed with an equal volume of 2X Laemmli sample buffer while pellets were reconstituted into 1X Laemmli sample buffer for Western analysis. Aliquots were separated on a 15% acrylamide gel at 50mAmps and transferred to nitrocellulose for 1 h at 0.6V in 25mM Tris, 192mM glycine, 15% methanol. Nitrocellulose was blocked with 1% BSA, 0.1% Tween 20, 5% goat serum, 150mM sodium chloride, 10mM Tris and then incubated in Anti-Xpress antibody (1:5000 in blocking solution) (Invitrogen) for 1 h at room temperature. The blot was then washed three times for five min with 0.05% Tween, 150mM sodium chloride and 10mM Tris, pH 7.4.
Subsequently, the blot was incubated in peroxidase labeled anti-mouse IgG (1:1000 in blocking solution) for 1 h at room temperature. After washing the blot four times for five min each, TMB Membrane Peroxidase Substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used to visualize the antibody binding.

The cell pellet was resuspended into 10mL of 6M guanidinium lysis buffer (Invitrogen), rocked for 10 min to ensure lysis, and centrifuged at 3000xg for 15 min to separate out particulate matter. Probond columns (Invitrogen) were prepared per the vendor's protocol and samples were loaded on each of the columns. Columns were washed and proteins were eluted following the pH elution protocol per the vendor's instructions (Invitrogen). Protein fractions were measured at 280nm.

Site-Directed Mutagenesis

Consensus PKC phosphorylation sites in the odorant and glutamate receptors were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) per the vendor's protocol with the following PCR program: 95°C for 1 min, 55°C for 1 min, 68°C for 12 min for 1 cycle and then 15 cycles with 95°C for 30 s, 55°C for 1 min, 68°C for 12 min. Products were digested with Dpn I restriction enzyme for 1 h at 37°C and 1μL of each product was transformed into Epicurian Coli XL1-Blue Supercompetent cells per the vendor's protocol (Stratagene). Colonies were screened for plasmids with inserts as described earlier and positive plasmids were sequenced using
Fidelity Sequencing kit (Oncor) to confirm mutagenesis of the desired nucleotide.

**Hydrophilic Loop Amplification**

Hydrophilic loops between two hydrophobic domains of the receptors that contained a consensus phosphorylation site for PKC were amplified. Specific primers near the loops for each receptor type were used in the following PCR program: 90°C for 5 min, 95°C for 1 min, 48°C (or 54°C for glutamate receptors) for 1 min, 72°C for 1 min for 40 cycles. This PCR program was performed on the original odorant and glutamate receptors as well as the mutagenized forms. An aliquot of the PCR product was electrophoresed on a 2% agarose gel and the amplified DNA (approximately 150 bp for odorant or 100 bp for glutamate receptors) was excised and gel purified using the QIAEX II Gel Extraction kit (Qiagen, Chatsworth, CA). This product was ligated into pCR2.1 (Invitrogen, San Diego, CA), transformed into DH5α competent cells, and plated on LB plates with 50μg/mL ampicillin and 1mg/plate Xgal. Plasmids with inserts were identified by restriction digestion with EcoRI. Positive plasmids were grown in LB broth cultures and purified using columns from Qiagen (Chatsworth, CA) per the vendor’s protocol. Products were sequenced on both strands using the Fidelity DNA Sequencing system (Oncor, Gaithersburg, MD) and were identified by comparison to previous sequence data.
Protein Expression in His-Patch ThioFusion Expression System

Positive inserts of the hydrophilic loop DNA were ligated into the appropriate pThioHis expression vector at 22°C for at least 16 h. Ligations were transformed and screened as described earlier. Sequence analysis as previously described was used to ensure the insert was in-frame with the expression vector and that the insert was in the proper orientation. LB broth culture aliquots were then used in a pilot expression study to determine expression levels and solubility of the protein. Cell growth was monitored at 550nm until the OD was approximately 0.5. Protein expression was induced with IPTG (final concentration 1mM) and hourly aliquots were taken for 3 h to monitor the expression. For each aliquot, cells were pelleted and the supernatant was removed. Pellets were resuspended in 50µL of lysis buffer (20mM Tris-HCl, pH 8, 2.5mM EDTA, 5mM imidazole and 250mM NaCl) and incubated with 50µg of lysozyme for 5 min. Samples were boiled for 90 s and centrifuged for 10 min to separate the pellet from the supernatant. Supernatants were transferred to a fresh tube and mixed with an equal volume of 2X Laemmli sample buffer and pellets were reconstituted into 1X Laemmli sample buffer for analysis. Aliquots were analyzed by Western analysis as described earlier using the Anti-Thio antibody (Invitrogen).

Purification of Fusion Protein

After protein expression, cells underwent osmotic shock to purify the fusion proteins. Once protein expression was complete and the supernatant
was removed, cell pellets were resuspended in 20mM Tris-HCl, pH8, 2.5mM EDTA, 20% sucrose, 100μg/mL Phenylmethlsulfonylfluoride (PMSF) and incubated on ice for 10 min. The cells were pelleted, the supernatant was removed, and the cells were resuspended in 20mM Tris-HCl, pH8, 2.5mM EDTA, 100μg/mL PMSF. After incubation on ice for 10 min, the mixture was centrifuged for 10 min to separate the soluble and insoluble fractions. Supernatants were removed and cell pellets were resuspended in 20mM Tris-HCl, pH8, 2.5mM EDTA, 100μg/mL PMSF. Aliquots of each sample were mixed with an equal volume of 2X Laemmli sample buffer for Western analysis to determine which fraction contained the fusion protein.

Sample fragments that had undergone osmotic shock were separated by electrophoresis on 15% acrylamide gels at 50mAmps and stained with GELCODE Blue Stain Reagent (Pierce, Rockford, IL). Fusion proteins were excised and eluted from the gel using the Centrilutor (Millipore, Bedford, MA) per the vendor’s protocol at 500V for at least 8 h. Eluted proteins were concentrated using Centricon-10 (Millipore) per the vendor’s protocol. Protein concentrations were determined using a Bradford assay with BSA as the standard.

Due to a potential phosphorylation site present in the leader sequence of the fusion protein, Enterokinase Max (EKMax) (Invitrogen) was used to cleave the leader sequence from the protein of interest. Each fusion protein was incubated overnight at 4°C with 1U of EKMax in 50mM Tris-HCl, pH 8,
1mM CaCl₂, 0.1% Tween 20. Digested products were separated on a 18% acrylamide gel and stained with GELCODE Blue Stain Reagent (Pierce). Protein products of the appropriate size were excised from the gel and eluted overnight as described earlier. Gel purified proteins were concentrated using Centricon-3 and underwent one buffer exchange into Assay Dilution Buffer (ADB) (20mM MOPS, pH 7.2, 1mM DTT, 1mM CaCl₂).

**Phosphorylation Assay**

Purified recombinant (> 95% purity) protein kinase CβII and protein kinase Cδ were obtained from Upstate Biotechnology (Lake Placid, NY), aliquoted, and frozen at -80°C. Immediately prior to use, enzymes were diluted tenfold with enzyme dilution buffer (Upstate Biotechnology). [γ-³²P]ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and immediately prior to the assay was diluted ten-fold into ADB with 75mM magnesium chloride and 500μm ATP (Sigma, St. Louis, MO). L-α-Phosphatidyl-L-serine, dioleoyl (Sigma), and the diacylglycerol 1-oleyl-2-acetyl-sn-glycerol (OAG) were diluted into chloroform and dried in a glass tube. The lipids were then solubilized in 3% Triton X-100 in ADB for a final concentration of 0.5mg/mL phosphatidylserine and 0.05mg/mL diacylglycerol. These components were used in a mixed micellar assay to measure protein kinase C phosphorylation (Hannun et al., 1985).

Assay conditions for PKCβ and δ were identical. For each enzyme, approximately 400ng of homogenous substrate was diluted with ADB to a final
volume of 30μL and added to 10μL lipids and 10μL of diluted [γ-32P]ATP. Substrates consisted of wild type and mutant forms of odorant and glutamate receptor phosphorylation sites. Blank tubes with no substrate were also run with each assay. For each assay, 25ng of each PKC subtype were added to the appropriate tubes and incubated for 25 min at room temperature. Reactions were stopped by either the addition of 3 volumes of 10% Trichloroacetic acid (TCA) or 1 volume of 2X Laemmli buffer. Samples stopped with TCA were incubated on ice for 20 min and an aliquot was blotted onto filter paper. Filters were washed three times with 5mL of 10% TCA and once with 5mL of ethanol under vacuum. After drying, the filters were mixed with EcoLite scintillation cocktail (ICN, Costa Mesa, CA) and counted on a Beckman LS6000IC counter (Fullerton, CA). Reactions stopped by 2X Laemmli buffer were boiled for 3 min and were separated by electrophoresis using a 15% acrylamide gel. Gels were dried and exposed to imaging film at -80°C for at least 16 h.

4.3 RESULTS

Protein Expression in Xpress System

Initial experiments utilized PCR products of odorant and metabotropic glutamate receptors obtained from olfactory receptor neurons. These large DNA fragments were subcloned into the appropriate pTrcHis expression vector so the inserts were in-frame with the N-terminal fusion peptide. This fusion construct encoded for an ATG translation initiation codon, an enterokinase
cleavage recognition sequence, an antibody epitope recognizable by the Anti-
Xpress antibody (Invitrogen), and six histidine residues in series that serve as
a metal binding domain (See Figure 4.1). This metal binding domain binds to
the ProBond resin (Invitrogen) and allows purification through immobilized
metal affinity chromatography. These fusion proteins were expressed and
purification using ProBond resin was attempted. ProBond columns bind the
hexa-His tag on the fusion protein until protein elution by low pH or high
imidazole concentration.

5'-ATG START-(HIS)_6-ENTEROKINASE SITE-Eco RI-INSERT-3'
ANTIBODY EPITOPE

Figure 4.1. Vector map of leader sequence from the pTrcHis vector. Included
in this leader sequence was the ATG start translation site, the hexa-his metal
binding tag which allowed the fusion protein to bind to the ProBond resin, the
antibody epitope for the Anti-Xpress antibody that allowed for visualization of
the fusion protein through Western analysis, an enterokinase site that allowed
for the cleavage and removal of the leader sequence from the protein of
interest, and an EcoRI site that allowed for the ligation of the insert which
contains the consensus phosphorylation site for each receptor.

Due to the high degree of protein insolubility, it was not possible to
purify the fusion proteins obtained from the expression of the PCR products.
Despite using denaturing conditions, the proteins were too hydrophobic to
interact with the ProBond resin in the column. There was never significant
binding to the resin and the fusion proteins were lost in initial binding steps of
the purification procedure. These results were not unexpected since
hydrophobic proteins such as 7TMD receptors often form insoluble aggregates (Thomas & McNamee, 1990).

**Site-Directed Mutagenesis**

Potential PKC phosphorylation sites for the odorant and glutamate receptors were mutated using the Quik-Change Mutagenesis kit (Stratagene). The potential phosphorylation sites correlated to the generalized consensus PKC phosphorylation site: \((R/K)_{1,3}-(X)_{2,0}^{\text{S/T}}-(X)_{2,0}-(R/K)_{1,3}\) in which \(X\) represents any amino acid (Pinna & Ruzzene, 1996). A serine/threonine residue on each of the PCR products was changed to alanine to abolish the potential phosphorylation site for protein kinase C. See Figure 4.2 for specific changes.

**Hydrophilic Loop Amplification**

Due to problems with protein insolubility in initial experiments, it was necessary to remove the hydrophobic transmembrane domains from the receptor proteins in an attempt to increase protein solubility. This reduction in protein size and hydrophobicity were predicted to increase protein solubility and facilitate purification. Consensus PKC phosphorylation sites were determined for each receptor type and those sites were amplified. See Figure 4.3 for specific areas of amplification. The new fusion proteins obtained using these small PCR products lacked any significant hydrophobic regions which would increase protein solubility as compared to the fusion proteins obtained from the entire PCR products.
Figure 4.2. Mutations of consensus phosphorylation sites for odorant receptor (panels A-C) and glutamate receptor (panels D-F). Bolded letters indicate consensus phosphorylation sites and roman numerals indicate transmembrane domains (see panels A & D). Panels B and E show the nucleotide and complementary sequence of the wild type portion of the receptor that contains the phosphorylation sites. Panels C and F include the mutant primers used to change the phosphorylation site to alanine. The nucleotide changes are underlined.
A. 

IV
TKEAMTLIVVITWSITTTIALLVALITRLSFCRSVIIINNYFCDHGPIVI

V
LACNDKFHINRVAIGCFVVLDCVPFFLLIVSICYCIGIAALLNISHGLERRK

VI
AMKTCTSHILIVALFVLPFIGTNSLTSSINANDRIINSSLTQIIIPMLNPFI

B. 

ALITRLSFRC
5'-GCTCTGATTACCAGACTGTCCTTTTGTAGATC-3' Nucleotide sequence
3'-CGAGACTAATGGCTCGACAGGAAAACATCTAG-5' Complement sequence

C. 

ALITRLAFCR
3'-CGAGACTAATGGCTCGACAGGAAAACATCTAG-5' Primer 1
5'-GCTCTGATTACCAGACTGTCCTTTTGTAGATC-3' Nucleotide sequence
3'-CGAGACTAATGGCTCGACAGGAAAACATCTAG-5' Complement sequence
5'-GCTCTGATTACCAGACTGTCCTTTTGTAGATC-3' Primer 2

D. 

SSRDVCYIIILAFGLFYICPFTLIAHRPTVISCYQLRLVLGSSAMCYSALVTKTNRIAR

ILAGSKKKICTRKHFSMSAWAQVVIAPILISLQLTEVLVILEPPEPIKSYPESIREAY

LICNTSTLGMVAPLGYSLILLSCTYYAFKTR

E. 

GSKKKIKICTRKHS
5'-GGCAGTAAAAAGAGATCTGCACTGGAAGCAGACAGC-3' Nucleotide seq.
3'-CCGTCACTTTTCTTCTAGACGCGTTCTGTGCG-5' Complement seq.

F. 

GSKKIARKHS
3'-CCGTCACTTTTCTTCTAGACGCGTTCTGTGCG-5' Primer 1
5'-GGCAGTAAAAAGAGATCTGCACTGGAAGCAGACAGC-3' Nucleotide seq.
3'-CCGTCACTTTTCTTCTAGACGCGTTCTGTGCG-5' Complement seq.
5'-GGCAGTAAAAAGAGATCTGCACTGGAAGCAGACAGC-3' Primer 2
Protein Expression and Purification in His-Patch ThioFusion System

The cDNA encoding for the hydrophilic loops from each receptor were ligated into the His-Patch ThioFusion expression vector, transformed, and protein expression was induced with IPTG. The thioredoxin moiety appears to increase the solubility of previously insoluble proteins. This fusion vector is very similar to the pTrcHis vector described earlier and illustrated in Figure 4.1, including the presence of a metal binding domain within the thioredoxin protein to allow for purification using ProBond resin. Native thioredoxin also has a tendency to localize at adhesion zones, areas on the cytoplasmic side of the inner membrane of the cell. This allows for protein purification to be performed by osmotically shocking the cells. During osmotic shock, the thioredoxin fusion protein may be released into the media from these adhesion zones.

Due to the insolubility of the fusion proteins created from the phosphorylation sites on the receptors, ProBond columns could not be used to purify the protein. Despite their insolubility, it was possible to purify the proteins with osmotic shock. This procedure induced the cells to release extraneous proteins and the fusion protein of interest was separated from other proteins on a 15% acrylamide gel. The fusion proteins (approximately 21kDa for odorant receptor fusion proteins and 18kDa for glutamate receptor fusion proteins) were excised and eluted from the gel. Protein concentrations for each of the fusion proteins for both wild type and mutant receptors were determined by Bradford assay. These fusion proteins were then incubated with
Figure 4.3. Primer sets used to amplify hydrophilic loop domain containing consensus phosphorylation sites in odorant receptor (panel A) and mGluR1 (panel B). Bolded letters indicate the phosphorylation sites and underlined letters indicate primers. Transmembrane domains are indicated by roman numerals.
EKMax to remove the leader sequence (approximately 12kDa), which contained a potential phosphorylation site for PKC. After incubation, the protein of interest was separated from the leader sequence of the protein and was purified by elution from the gel. These proteins, which were approximately 9kDa for odorant receptors and 5.5kDa for glutamate receptors, were used in the phosphorylation assay.

**Phosphorylation Assay**

In order to test the efficacy of the PKC subtypes used, histone type III, a known substrate for PKC was used as a positive control. A mixed micellar assay (Hannun et al., 1985) was used to test the specific activity of 25ng PKC on 10μg of histone. Samples were mixed and allowed to react for 30 s before termination with 10% cold TCA. After precipitation, samples were spotted on filters for washing and counting. Triplicates were run for each sample and the specific activity for each subtype was determined. PKCβ had a specific activity of 0.124 +/-0.017 nmol phosphate/μg histone/min while the specific activity of PKCδ was 0.196 +/- 0.014 nmol phosphate/μg histone/min. This control assay confirmed that the PKC subtypes were active and able to phosphorylate substrates.

Proteins generated from the hydrophilic loops of odorant receptors and glutamate receptors were analyzed with their mutant counterparts in assays that measured phosphorylation by both PKCβII and PKCδ. In both cases, mutant receptors were not phosphorylated by either kinase subtype. Odorant
Figure 4.4. Autoradiographs of receptor phosphorylation assays. Lane W for each panel represents wild type substrate and lane M represents mutant substrate. Arrows indicate phosphorylated substrate based on the molecular weight of the substrate. Panel A depicts phosphorylation of odorant receptor substrates by PKCβ and panel B shows phosphorylation of odorant receptor substrates by PKCδ. Panel C shows phosphorylation of glutamate receptor substrates by PKCβ and Panel D shows PKCδ phosphorylation of glutamate receptor substrates.
receptors were phosphorylated by PKCδ but not PKCβ, while glutamate receptors were phosphorylated by both. In the case of glutamate receptors, PKCβ gave a stronger signal than the PKCδ subtype (Figure 4.4).

4.4 DISCUSSION

Previous work in olfactory receptor neurons in the channel catfish have shown that multiple subtypes of PKC are present. Specifically, PKCβ, a member of the conventional PKC subtypes and PKCδ, a member of the novel PKC subtypes were shown to be expressed in olfactory neurons. Since conventional PKC subtypes require calcium and novel PKC subtypes do not, in vitro assays with isolated olfactory cilia were able to utilize the subtypes' unique activation requirements to determine the most likely candidate involved in odor induced phosphorylation. This study found that odor induced phosphorylation was abolished in the presence of a specific inhibitor of calcium sensitive PKCs and concluded that PKCβ was the most likely candidate to be involved in this process (Bruch et al., 1997a).

The work done in this study began determining in vitro, the specific interactions between PKC and 7TMD receptors present in the olfactory receptor neurons. Based on the previous in vitro PKC study (Bruch et al., 1997a), it was postulated that PKC phosphorylated activated 7TMD receptors in the olfactory neurons and played an instrumental role in their desensitization. Within this context, two types of 7TMD receptors known to be present in olfactory neurons, the putative odorant receptor and the
metabotropic glutamate receptor (mGluR), were used to test this hypothesis. Consensus phosphorylation sites (Pinna & Ruzzene, 1996) within the amplified regions of the PCR products were determined (see Figure 4.2) and proteins containing these phosphorylation sites were produced. For each receptor subtype, only one consensus phosphorylation site was found within the region of the receptors amplified by RT-PCR. The expressed proteins were used as substrates in mixed micellar assays to determine if PKCβ or PKCδ were able to phosphorylate the receptors at their respective phosphorylation sites.

This study demonstrated that the consensus site from the mGluR substrate can be phosphorylated by both PKCβ and PKCδ, although the signal for PKCβ was stronger (see Figure 4.4). This differential phosphorylation by the two subtypes of PKC may be indicative of their physiological role in vivo. Group I mGluRs’ primary mode of action is to stimulate phospholipase C activity which ultimately causes an increase in intracellular calcium. This action can then trigger the calcium sensitive PKC activity which results in phosphorylation and desensitization of the mGluR. Since there are no known interactions between GRKs and mGluRs (Gereau & Heinemann, 1998), the role of PKC in the desensitization of these receptors may be critical. Based on the differential phosphorylation of the two subtypes tested, a plausible hypothesis would be that PKCβ may be an integral part in the negative feedback loop that turns off mGluR1 while the role of PKCδ may be less important.
The consensus phosphorylation site of the mGluR tested resides within the second intracellular loop of the receptor, an important site for interaction with G proteins and a known area of PKC phosphorylation in Group I mGluRs (Francesconi & Duvoisin, 1998; Gereau & Heinemann, 1998). Other work has shown that while deleting C694-T695 residues did not result in a nonfunctional receptor, the ability of the receptor to cause cAMP accumulation was significantly reduced. Taken with other results, the authors concluded that mGluRs have more than one domain that interacts with G protein α subunits and that C694-T695 are key components, along with others, for these interactions to occur. These residues appear to interact with Gs and are involved in activating the accumulation of cAMP, a secondary function of Group I mGluRs (Francesconi & Duvoisin, 1998). Another study has shown that the intracellular loop 2 domain of mGluR1 plays a critical role in the activation of a PLC-coupled G protein. Specifically, the authors found that the 16 C-terminal residues of intracellular domain 2 were necessary for optimum coupling to PLC (Gomeza et al., 1996).

Interestingly, T695 is the threonine that is phosphorylated by PKCβ and PKCδ within the consensus phosphorylation site tested in this study. This phosphorylation site is also contained within the 16 C-terminal residues of intracellular domain 2 which were shown to be critical for coupling to PLC (Gomeza et al., 1998). Taken with the results from other studies (Gomeza et al., 1994; Francesconi & Duvoisin, 1998), this study predicts that the
consensus site within intracellular domain 2 is a functional phosphorylation site on the receptor and that it may be phosphorylated during the process of desensitization in vivo. Another study (Gereau & Heinemann, 1998) analyzed PKC phosphorylation of mGluR5 through mutations of 24 PKC phosphorylation sites, although it was unclear from the authors' descriptions whether this particular phosphorylation site was included in their study. The authors found that critical PKC phosphorylation sites were grouped within intracellular domain 1 and 2 as well as a portion of the carboxyl intracellular tail of the mGluR (Gereau & Heinemann, 1998). These results correlate with the conclusion that intracellular domain 2 is an important site of interaction for mGluRs.

The consensus phosphorylation site found on odorant receptors was found on all of the odorant receptor PCR products amplified in an earlier study (Medler et al., submitted). This was the only consensus phosphorylation site for PKC (Pinna & Ruzzene, 1996) found within these products and inspection of the sequence from full length clones of catfish odorant receptors (Ngai et al., 1993b) found that this was the only phosphorylation site found in a majority of the receptors. Inspection of odorant receptors from rat (Buck & Axel, 1991) failed to find any region of the receptors that consistently contained consensus phosphorylation sites for PKC. A few of the rat receptors had phosphorylation sites on the carboxyl terminal tail (personal observation).

Phosphorylation of the consensus site tested from the odorant receptors found that PKCδ but not PKCβ phosphorylated this site. This was a surprising
result due to earlier *in vitro* studies in catfish olfactory neurons that indicated odor induced phosphorylation was caused by PKCβ (Bruch et al., 1997a). Since there are no other consensus phosphorylation sites available for PKCβ on the receptor, it is probable that PKCβ is interacting with other components of the signal transduction pathway and not with the receptor. Which components are being phosphorylated by this PKC are purely speculative at this point, but known substrates for PKC within this pathway include phospholipase C, adenylyl cyclase, IP₃ receptor, G-protein coupled receptor kinase, and dynamin (Liu, 1996; Pronin & Benovic, 1997).

This study shows for the first time in any system the novel observation of a potential extracellular phosphorylation site on a 7TMD receptor. The consensus phosphorylation site used in this study was located on a hydrophilic loop between transmembrane domain IV and transmembrane domain V. Based on current knowledge of the structure of 7TMD receptors, this hydrophilic domain comprises extracellular loop 2. Results from this study indicate that this is a PKC consensus phosphorylation site, but what role phosphorylation at this site plays is unclear. However, there are two times within the life cycle of a receptor when the extracellular domains of the receptor are exposed to the internal components of the cell. One of these times is when the receptor is being expressed in the endoplasmic reticulum, processed through the Golgi apparatus, and carried in vesicles to the plasma membrane. Since 7TMD receptors are maintained within the membrane in their final
conformation during these processes, the extracellular domains are in contact with the intracellular environment. It is possible that phosphorylation of the receptors during this processing is necessary for proper targeting of the receptor to occur. The other time the extracellular domains of the receptor are exposed to the internal components of the cell is during the process of desensitization. After 7TMD receptors undergo phosphorylation and deactivation, they are internalized by clathrin coated vesicles to be recycled to the membrane or degraded. During this process, the receptor's extracellular domains are again exposed to the cell's internal environment (Ferguson et al., 1997). Both of these processes are not associated with acute increases in intracellular calcium. For this reason, phosphorylation by PKCβ would not be feasible due to a lack of high calcium levels in the cell. Instead, the novel PKC subtype, PKCδ, which does not depend on calcium for activation, is a more likely candidate for phosphorylation in these processes. It is possible that receptors must be phosphorylated during this process in order for the cell to determine if recycling or degradation should occur. Additional work is needed to determine the novel role of this extracellular phosphorylation site on odorant receptors.
CHAPTER 5. CONCLUSIONS AND PERSPECTIVES

This dissertation research further characterized the expression of 7TMD receptors in olfactory receptor neurons. This work addressed odorant receptor expression in individual olfactory neurons, discovered the expression of mGluR in olfactory neurons, and provided in vitro information on the potential regulation of these receptors by phosphorylation.

The first specific aim of this dissertation addressed the expression of odorant receptors in olfactory neurons. This question has been studied using different methodologies which have resulted in conflicting data. Electrophysiological data have shown that neurons can respond to multiple stimuli, providing indirect evidence that a neuron expresses multiple receptors (Ivanova & Caprio, 1993). In situ hybridization studies of molecularly characterized receptors have provided indirect evidence that a neuron only expresses one receptor (Ngai et al., 1993a; Vassar et al., 1993). This question has received so much attention because it is thought that receptor expression in the neurons is key to understanding coding of odorants in the brain and that coding of odor stimuli in the brain is a key point in understanding how olfaction functions.

Chapter two presents results to begin resolving the disparity between electrophysiology and in situ hybridization data. It seemed that a direct measure of receptor expression in olfactory neurons was necessary to answer
the question of how many odorant receptors are present in individual receptor neurons. The present study found that individual olfactory receptor neurons can express more than one receptor gene and that a majority of neurons tested expressed at least two receptors. These findings support the electrophysiological data that find many neurons are responsive to multiple stimuli. These findings do not agree with conclusions based on *in situ* hybridization data (Ngai et al., 1993a; Vassar et al., 1993), which may mean that inappropriate conclusions have been drawn from those data.

One possible explanation for these findings is that there are more receptors present in the olfactory epithelium than first thought. Even if a species is only thought to detect 100 odors, it may still possess hundreds of receptors. It is also difficult to test with certainty all the odors that are detected by a species, so it is possible that a more complicated odor repertoire exists for a particular species than first appreciated.

The presence of multiple receptors in single neurons may not necessarily indicate that a particular neuron can detect more than one odor. It is possible, though not probable, that even though receptors are being expressed from different genes, they are all receptors for the same ligand. It is also possible that these receptors can all recognize the same molecule, but recognize different structural features of that molecule. This would mean that all the receptors are functioning as the same odorant detector. However, there is no evidence to date that indicates what ligand these receptors bind and
electrophysiological evidence supports the hypothesis that multiple receptors allow a neuron to be responsive to multiple stimuli.

With the determination that olfactory neurons in a teleost can express multiple receptors, it seems unlikely that the simplest model of odorant coding is valid. The model developed for mammals states that a given odor stimulus is recognized in the brain by the identity of the stimulated peripheral neuron. This model has been supported by in situ hybridization data which has shown a given receptor is only expressed in a small subset of neurons, implying that due to the small number of odorant receptors present, there was likely only one receptor expressed in a given neuron (Ngai et al., 1993a; Vassar et al., 1993). Based on the results of the current study, it seems more likely that odorant coding must include some integration of signals at the olfactory bulb and cortex. Further work is needed to characterize the role of multiple receptors within olfactory neurons before odorant coding by the olfactory bulb can be fully understood. One possible approach to begin addressing the role of odorant receptors in coding of stimuli would be to use in situ hybridization of multiple odorant receptors at the axon terminals of the olfactory neurons, since it has been shown that odorant receptor mRNA is found in receptor cell axon terminals (Vassar et al., 1994; Wang et al., 1998). It is currently unknown if there are multiple receptors present at the terminals, even though the present study has now shown that multiple receptors are present in the neurons. If odorant receptors target the neuron axon to a specific glomerulus (Wang et al.,
1998), then it would be interesting to know if all the receptors expressed in a single neuron are expressed at the termini as well. The answer to this question will begin defining the role of these receptors in coding as well as determine which coding model is accurate.

A secondary issue addressed in chapter two is the expression of odorant receptors in taste epithelium of the channel catfish. Primers specific to odorant receptors were used to amplify receptors very similar to odorant receptors from the catfish barbel. Further analysis by RT-PCR of taste buds indicated that these receptors were expressed in the taste bud, implying a possible role for these receptors as putative taste receptors. While the expression of these receptors in the taste system needs to be further characterized before they can be called taste receptors, this work is not without precedent from other systems (Abe et al., 1993a; Abe et al., 1993b; Matsuoka et al., 1993; Thomas et al., 1996). These results also lend support to the hypothesis that the odorant receptors may belong to an even larger group of 7TMD receptors that act as chemoreceptors in various cells. Receptors very similar to odorant receptors have now been found in numerous tissues outside the olfactory epithelium (Nef & Nef, 1997; Thomas et al., 1996; Vanderhaeghen et al., 1993; Walensky et al., 1998), and it is possible that these receptors serve a broader function beyond odor detection. The specific function of the receptor may be determined by the cell in which it is expressed.
The second aim of this dissertation was to characterize the novel observation that mGluRs are present in olfactory neurons. While Pang et al. (1994) reported that a mGluR was present in the olfactory epithelium of Atlantic salmon, they did not determine which subtype was present or where it was expressed in the olfactory epithelium. In the present study, it was shown that there are at least two subtypes, mGluR1 and mGluR3, present in the olfactory epithelium and that these receptors are expressed in olfactory receptor neurons. Additionally, it was shown that these receptors are coexpressed with each other and that they are coexpressed with olfactory receptors in the same olfactory neurons. Further, it was shown for the first time that the receptor proteins for the mGluRs are localized at the dendritic knob and cilia of the neurons, the area of the cell that interacts with odorants. Finally, electrophysiological evidence found that antagonists specific to these mGluR subtypes significantly inhibited the olfactory response to glutamate.

Taken together, these results suggest a role for mGluRs in olfaction, although their exact function requires further study. The receptors are localized at the correct site in order to have some role in olfaction and the electrophysiological evidence shows that these receptors can modulate olfactory responses. The most obvious role for mGluRs is to function as the glutamate odorant receptor and evidence from this dissertation indicates that it is likely that the mGluRs contribute to the glutamate response. However, it seems unnecessary to have at least two subtypes present that function through
different second messenger systems, if the primary function of these receptors
is to detect the odorant glutamate. Since the electrophysiological evidence
indicates that mGluR antagonists also reduce the response to the odorant
methionine, a wider role for mGluRs beyond the detection of glutamate
odorants is possible. However, this observation may not be significant due to
the structural similarities between these antagonists and glycine, which is
known to partially cross react with the methionine receptor (Caprio & Byrd,
1984; Bruch & Rulli, 1988).

Another possible role for mGluRs may be to modulate the odorant
response due to stimulation of odorant receptors. Currently, there is no known
source of glutamate that could interact with these receptors at the dendritic
knobs and cilia except when the odorant glutamate is present. However, this
does not mean that there is not a source of glutamate that has not yet been
detected. Other work has shown that ionotropic glutamate receptor subunits
are also present in the dendritic knobs of rat olfactory neurons (Thukral et al.,
1997). This lends further support to the hypothesis of the presence of an
unknown source of glutamate that interacts with the neurons at the site of
odorant interaction. Further electrophysiological characterization of these
mGluRs is needed. A full range of the effects of mGluR antagonists on the
responses of odor stimuli would be useful to make a determination as to the
possible role for mGluRs in olfaction.
The presence of mGluRs in olfactory receptor neurons lends support to the hypothesis that multiple types of 7TMD receptors may be present within the neurons. Future work may characterize the presence of other non-odorant 7TMD receptors in olfactory neurons, such as the putative pheromone receptors. An attempt to characterize the presence of pheromone receptors in these neurons was made, but without success. It may be that the pheromone receptors present in catfish differ too significantly from the putative pheromone receptors that have been characterized (Dulac & Axel, 1995) and were not detected. It is also possible that the pheromone receptors were not being expressed in the olfactory neurons of the catfish tested, as all the neurons used in these studies were obtained from animals that were relatively immature. These animals would not be expected to actively detect pheromones.

The final aim of this dissertation addressed the regulation of these receptors by protein kinase C (PKC). *In vitro* phosphorylation assays of a consensus site for PKC on these receptors found selective phosphorylation by the PKC subtypes present in olfactory neurons. Earlier work had shown that PKCβ and PKCδ are expressed in the olfactory receptor neurons (Bruch et al., 1997a). The glutamate receptor was phosphorylated by both PKC subtypes, PKCβ and PKCδ, but was more intensely phosphorylated by PKCβ. The odorant receptor was only phosphorylated by PKCδ. These selective
phosphorylations of the receptors indicate that receptor deactivation can be differentially controlled by specific PKC subtypes.

mGluRs have been shown to be phosphorylated by PKC (Gereau & Heinemann, 1998) at the second intracellular loop, the site of the consensus phosphorylation site tested. This site has also been shown to play an important role in coupling the receptor to adenylate cyclase and phospholipase C (Gomeza et al., 1996; Francesconi & Duvoisin, 1998). It seems likely that this phosphorylation site functions in vivo and it is an important site involved in desensitization.

In odorant receptors, the novel observation was made that the only consensus phosphorylation site for PKC was present on extracellular loop 2. An extracellular PKC phosphorylation site has apparently not been previously reported in 7TMD receptors. This PKC consensus site was phosphorylated by the calcium independent PKCδ, but not PKCβ, which had been shown to be responsible for odor induced phosphorylation (Bruch et al., 1997a). This suggests that this phosphorylation site may be used as a targeting signal during protein processing or down regulation. Future studies are needed to determine the function of this extracellular phosphorylation site. An initial study to perform would involve the heterologous expression of a full length receptor. Mutation of this phosphorylation site would determine if the receptor could still be properly targeted to the membrane. Using this same system, it would also be possible to determine if the receptor was properly processed and recycled.
during desensitization. Studies of this type would allow determination of the function of an extracellular phosphorylation site.
REFERENCES


116

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Medler, K., Tran, H., and Bruch, R. (submitted). Odorant receptor expression in single olfactory receptor neurons and taste buds from the channel catfish, *Ictalurus punctatus*.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


APPENDIX

John Wiley & Sons, Inc.
Publishers Since 1807

March 27, 1998

Kathryn Medler
Department of Biological Sciences
Louisiana State University
Baton Rouge, LA 70803
VIA FAX ONLY: 504-388-2597

Dear Ms. Medler:

RE: Your request to reuse your paper from JOURNAL OF NEUROBIOLOGY (in press), (ISSN 0022-3034).

Thank you for your request of March 18, 1998. Please be aware that although you assigned rights to this work to us in your contributor agreement, you retained the following non-exclusive print rights:

1. Proprietary rights, other than copyright, such as patent rights.
2. The right to make copies of all or part of the work for your use in classroom teaching.
3. The right to use, after publication, all or part of this material in a book by you or in a collection of your works.
4. The right to make copies of the work for internal distribution within the institution where you work.
5. The right to use figures and tables from the work, and up to 250 words of text, in print media only.
6. The right to make oral presentation of the material in any forum.

You must include a credit notice with the following information: Title, author(s), journal title, Copyright © (year and owner).

If you have any questions, please call me at (212) 850-6014.

Sincerely,

Patrick Murphy
Permissions Assistant

VISIT OUR WEBSITE @ "HTTP://WWW.WILEY.COM" FOR PERMISSIONS INFORMATION AND REQUEST FORMS
Kathryn Medler obtained a bachelor of science degree in biology from Texas A&M University in 1989. As an undergraduate, Kathryn characterized the effects of estrogen on vitellogenin levels in Kemp's Ridleys sea turtles in the laboratory of Dr. Duncan MacKenzie. Kathryn also worked as a student worker for Dr. David Owens and was responsible for the care and maintenance of 11 sea turtles. Kathryn married and moved to San Diego, California, where she obtained a master of science degree in biology with a concentration in physiology from San Diego State University in 1992. Her research characterized the steroid levels in developing alligator embryos through sex determination and differentiation and was conducted at the Center for the Reproduction of Endangered Species of the San Diego Zoo under the supervision of Dr. Valentine Lance. She also worked at the center for Dr. Nancy Pratt as a part time research associate analyzing steroid hormone levels in iguanas. Kathryn then worked as a senior research assistant in Assay Development at Cytel Corporation under the supervision of Dr. Tom Pritchett. She moved to Baton Rouge, Louisiana, and began working on her doctoral studies under the supervision of Dr. John Trant characterizing the steroid enzyme levels in testes from the channel catfish. Dr. Trant moved to another university and Kathryn began working on characterizing G protein coupled receptors in olfactory neurons of catfish under the supervision of Dr. Richard Bruch. She will obtain the degree of Doctor of Philosophy in August, 1998.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Kathryn Medler

Major Field: Zoology

Title of Dissertation: Characterization of Seven Transmembrane Domain Receptor Gene Expression in Olfactory Receptor Neurons

Approved:

Richard Buck
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: May 20, 1998