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Bile salts as olfactory and gustatory stimuli in the channel catfish

Shane Howell Rolen
Louisiana State University and Agricultural and Mechanical College

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BILE SALTS AS OLFACTORY AND GUSTATORY STIMULI IN THE CHANNEL CATFISH

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

Shane Rolen
B.S., University of Louisiana at Monroe, 2000
M.S., Louisiana State University, 2002
December 2008
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<tr>
<td>7-TMD</td>
<td>7-transmembrane domain G-protein coupled receptor</td>
</tr>
<tr>
<td>Ala</td>
<td>L-alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>C3</td>
<td>carbon #3 in the steroid backbone of bile salt molecules</td>
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<tr>
<td>C7</td>
<td>carbon #7 in the steroid backbone of bile salt molecules</td>
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<tr>
<td>C12</td>
<td>carbon #12 in the steroid backbone of bile salt molecules</td>
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<td>C24</td>
<td>carbon #24 in the side chain of bile salt molecules</td>
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<tr>
<td>CA</td>
<td>cholic acid</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>CDC</td>
<td>chenodeoxycholic acid</td>
</tr>
<tr>
<td>CFTW</td>
<td>charcoal filtered tap water</td>
</tr>
<tr>
<td>CN</td>
<td>cranial nerve</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DCA</td>
<td>deoxycholic acid</td>
</tr>
<tr>
<td>EMRR</td>
<td>excitatory molecular receptive range</td>
</tr>
<tr>
<td>EOG</td>
<td>electro-olfactogram</td>
</tr>
<tr>
<td>FB</td>
<td>forebrain</td>
</tr>
<tr>
<td>GBS</td>
<td>glycine-conjugated bile salts</td>
</tr>
<tr>
<td>GCA</td>
<td>glycocholic acid</td>
</tr>
<tr>
<td>GCDC</td>
<td>glycochenodeoxycholic acid</td>
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<tr>
<td>Glu</td>
<td>L-glutamate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>IMP</td>
<td>inositol 5’-monophosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5 triphosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>inositol 5’-triphosphate</td>
</tr>
<tr>
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<td>lithocholic acid</td>
</tr>
<tr>
<td>Met</td>
<td>L-methionine</td>
</tr>
<tr>
<td>mOR</td>
<td>molecular olfactory receptors</td>
</tr>
<tr>
<td>MS-222</td>
<td>ethyl-m-aminobenzoate methane sulfonic acid</td>
</tr>
<tr>
<td>NBS</td>
<td>non-conjugated bile salts</td>
</tr>
<tr>
<td>OB</td>
<td>olfactory bulb</td>
</tr>
<tr>
<td>ORNs</td>
<td>olfactory receptor neurons</td>
</tr>
<tr>
<td>Pro</td>
<td>L-proline</td>
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<tr>
<td>TBS</td>
<td>taurine-conjugated bile salts</td>
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<td>TCA</td>
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<tr>
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<td>taurodeoxycholic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>taurolithocholic acid</td>
</tr>
<tr>
<td>TLCS</td>
<td>taurolithocholic acid-3-sulfate</td>
</tr>
<tr>
<td>VNO</td>
<td>vomeronasal organ</td>
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ABSTRACT

A chemotopic map of biologically relevant odorants (that include amino acids, bile salts and nucleotides) exists in the olfactory bulb (OB) and forebrain (FB) of channel catfish, *Ictalurus punctatus* (Chapter one). Neurons processing bile salt odorant information lie medially within these bilaterally symmetric structures; however, information as to how single neurons discriminate and process this odorant information is lacking. Chapters two and three of the dissertation identify the range of odorant bile salt molecules that excite these neurons [i.e. the excitatory molecular receptive range (EMRR)] within the bile salt chemotopic zones of the OB and FB. The results of the investigations of single bile salt responsive neurons within the OB indicate that these neurons are selectively excited by combinations of molecular features found on the side-chain and the steroid nucleus of bile salt molecules. Further, the results of the investigations of single bile salt responsive neurons within the FB indicate that their EMRRs are virtually identical to that of OB neurons suggesting that little modification of the neural olfactory quality code for these molecules occurred between the OB and the FB.

Bile salts are known olfactory stimuli to teleosts, but only a single report (Yamashita et al. 2006) indicated that the taste system of a fish was sensitive to this class of stimuli. Chapter four investigates the gustatory sensitivity of the facial taste system to bile salts in the channel catfish. Bile salts were shown to be highly effective facial taste stimuli with estimated electrophysiological thresholds of approximately $10^{-11}$M-$10^{-10}$M. Multiunit cross-adaptation experiments indicate that bile salts and amino acids bind to relatively independent receptor sites; however, nerve twig data and a few single fiber recordings suggest that both independent and shared neural pathways exist for the transmission of bile salt and amino acid information to the primary gustatory nucleus of the medulla.
The findings of the present report aid in understanding how bile salt molecules are
detected and initially processed by the olfactory and gustatory systems in catfish and further
suggest that bile salt odorant information is not greatly transformed by central olfactory neurons
(Chapter five).
CHAPTER 1

INTRODUCTION
Teleosts possess two principle chemosensory systems, the olfactory and gustatory systems, with which chemical information about the surrounding environment is detected and relayed to the central nervous system (CNS). In fishes, olfactory and gustatory stimuli are water-soluble compounds present in the aqueous environment. Amino acids, bile salts, gonadal steroids, nucleotides and polyamines were identified previously as behaviorally relevant molecules that stimulate the olfactory system (Hara 1994; Sorensen and Caprio 1998; Michel et al. 2003; Rolen et al. 2003; Caprio and Derby 2008), while amino acids, nucleotides and quinine (to represent bitter substances) were shown to be behaviorally relevant stimuli for the gustatory system (Caprio et al. 1993; Lamb and Finger 1995).

OLFACTORY SYSTEM

Olfactory systems confer the ability to detect and discriminate a vast number of biologically relevant compounds which aid in identifying and locating food sources, conspecifics, mates and spawning habitats (Sorensen and Caprio 1998). Initially, the olfactory system discriminates odorants with an assortment of molecular olfactory receptors (mORs) located within the apical ciliary and microvillar membranes of olfactory receptor neurons (ORNs). Vertebrate mORs are members of the superfamily of seven-transmembrane domain (7-TMD) G-protein coupled receptors (Buck and Axel 1991). Although co-expression of a few ORs may occur in small populations of both mammalian (Rawson et al. 2000) and fish (Sato et al. 2007) ORNs, ORNs generally express one of ~1,000 mOR genes in mammals (Buck and Axel 1991) or one of ~100 in fish (Ngai et al. 1993; Barth et al. 1996) which encodes for receptor protein molecules. The majority of fish mORs, however, are orphan receptors; the lone exception is the basic amino acid receptor discovered in goldfish (Ngai et al. 1999). ORNs expressing a particular mOR are randomly scattered throughout the olfactory epithelium in catfish (Barth et al. 1996), have a nested expression in zebrafish (Baier and Korsching 1994) or
are segregated into one of four zones in mammalian olfactory epithelia (Buck and Axel 1991); however, in all these species, axons of ORNs expressing like ORs converge onto discrete areas in the olfactory bulb (OB), termed glomeruli, where they make synaptic contacts with dendrites of mitral cells.

Recent evidence indicates a correlation exists in the few species of teleosts investigated between the anatomical type of ORN, the type of molecular receptor expressed, the class of biologically relevant odorant detected, the particular transduction cascade activated and the portion of the OB that processes the specific type of odorant information (Friedrich and Korsching 1998; Hansen et al. 2003; Hara and Zhang 1996; Nikonov and Caprio 2001; Sato et al. 2005). Specifically for bile salts, these odorants are detected by ciliated ORNs that express OR-type of mORs that activate the $G_{olf}/cAMP$ transduction cascade and project primarily to the medial OB both dorsally and ventrally. Axons of OB output neurons that transmit this (Døving et al. 1980; Nikonov and Caprio 2001) and other classes of socially relevant odor information, such as that for putative pheromones (Kyle et al. 1987; Hamdani et al. 2000; Hamdani et al. 2001; Hamdani and Døving 2003; Lastein et al. 2006; Sorensen et al. 1991), comprise portions of the medial olfactory tract which project to medial regions of the olfactory forebrain (Bass 1981; Finger 1975). A recent electrophysiological investigation in channel catfish documented the excitability of forebrain (FB) neurons within this region to mixtures of bile salt stimuli (Nikonov et al. 2005). Yet, the ability of single neurons within the OB and FB of teleosts to discriminate individual bile salt molecules (i.e. social stimuli for fish) is unknown.

**GUSTATORY SYSTEM**

Taste can be described as the chemosensory information transmitted from peripheral taste receptors to the hindbrain via cranial nerves (CN) VII, IX and X. Peripheral receptors for tastants are expressed by taste cells localized to structures termed taste buds embedded in the
epithelia of the oropharyngeal cavity and, in the case of channel catfish, across the surface of the entire body which earned the catfish the nickname “the swimming tongue”. Taste receptor cells express T1R and T2R molecular receptors which are G-protein coupled receptors (Ishimaru et al. 2005); however, T1Rs function as dimers and detect amino and nucleic acids while T2Rs have not been shown to exist as dimers and detect bitter compounds (Oike et al. 2007). Taste receptor cells are different from olfactory receptor cells that are primary neurons in that taste cells are epithelial cells and not neurons and as such must synapse with specific cranial nerves to relay taste information to the central nervous system. Taste buds lying within the oropharyngeal cavity are innervated by CN IX and X while those positioned on the exterior surface of the body are innervated by CN IX (Herrick 1901; Atema 1971). Taste information is then transmitted to the dorsal parts of the facial and vagal lobes, the primary gustatory nuclei of the CNS (Herrick 1901; Atema 1971).

**BILE SALTS**

Over the past 30 years, the detection and processing of amino acid stimuli by chemosensory systems of teleosts has been well-studied (Sorensen and Caprio 1998; Caprio and Derby 2008). During this time, amino acids have garnered much attention as gustatory stimulants in teleosts and the sensitivity of the peripheral taste system to amino acids is known (Caprio et al. 1993; Hara 1994; Sorensen and Caprio 1998); however, knowledge of the response specificity of olfactory and gustatory systems of the same species to these stimuli is sparse considering the large number of extant teleost species (Hara 1975; Caprio 1978; Goh and Tamura 1980; Marui et al. 1983; Hara et al. 1999). To further add complexity to this situation, a recent electrophysiological investigation indicated that bile salts stimulate gustatory fibers of the facial nerve in rainbow bow trout (Yamashita et al., 2006) making it the first study to implicate gustation in the chemosensory detection of these compounds.
What are bile salts and why is this class of molecules important chemosensory stimuli to teleosts? Bile salts are biliary steroids derived from cholesterol, synthesized by the liver and stored in the gall bladder. These compounds are released from the gall bladder into the intestinal lumen and function to emulsify fats and subsequently aid in the absorption of lipids and fat-soluble vitamins (Haslewood 1967). Vertebrates recycle the majority of bile salts released into the intestine through enterohepatic circulation creating a continuous cycle where bile salt molecules are reused. Although most bile salts are reabsorbed by the enterohepatic system, in fishes some are released into the water column in feces and urine that could possibly function as odorant molecules. Teleosts, the largest group of extant vertebrates, synthesize a vast array of structurally diverse bile salt molecules: cyprinol sulfate, chimaerol, cholic acid, chenodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid (Denton et al. 1974; Tammer 1974; Zhang et al. 2001; Yeh and Hwang 2001; Thwaits et al. 2006). Some marine species also produce cysteinolic acid-conjugated bile salts (Une et al. 1991; Goto et al. 1996); however, related fish species of a given taxon generally produce the same types of bile salts (Tammer 1974). For instance, bile from channel catfish gall bladders contains 83% taurocholic acid, 15% taurochenodeoxycholic acid and 2% taurodeoxycholic acid (Kellogg 1975); and that blue catfish (*Ictalurus furcatus*) bile contains 84% taurocholic acid and 16% taurochenodeoxycholic acid. Stone catfish (*Noturus flavus*) synthesize a similar compliment of taurine-conjugated bile salts: 80% taurocholic acid, 14% taurodeoxycholic acid, 4% taurochenodeoxycholic acid and 1% taurolithocholic acid (PW Sorensen, personal communication).

The goal of this manuscript is two-fold: (1) to determine how olfactory neurons at the initial stages (OB and initial portion of the FB) process bile salt odorant information in channel catfish (Fig. 1) and (2) to determine whether the gustatory system of the animal is also sensitive
to these compounds. Chapters two and three describe the excitatory molecular receptive ranges (EMRR) of OB and FB neurons to bile salts, respectively. Chapter four indicates the sensitivity and specificity of the facial taste nerve to bile salts. The findings presented here aid in the understanding of the detection and processing of bile salt chemical information in channel catfish which help elucidate the behavioral relevance of this important class of social stimuli. This is the first study to describe the EMRRs of OB and FB neurons to bile salts in fishes, and one of only a few investigations of response specificity of single neurons within the forebrain of a vertebrate. The taste study in chapter four is only the second investigation to report gustatory responses to bile salts in fishes.

Chapters two and four were reprinted with permission from the Journal of Neurophysiology and the Journal of Experimental Biology, respectively. These articles were slightly modified for inclusion into the current manuscript: 1) the introductions were shortened by moving general background information to Chapter one and 2) figures illustrating the experimental setup were included for both chapters two and four.
Figure 1.1. The channel catfish, *Ictalurus punctatus*, the model system utilized in the present study.
CHAPTER 2

PROCESSING OF BILE SALT ODOR INFORMATION BY SINGLE OLFACTORY BULB NEURONS IN THE CHANNEL CATFISH*

* Reprinted by permission of *Journal of Neurophysiology*
INTRODUCTION

The arrangement of glomeruli within the glomerular layer of the OB is such that odorant molecules sharing similar molecular features tend to activate neighboring glomeruli resulting in a chemotopic map relating general chemical features to spatially confined OB regions (Xu et al. 2000). The existence of a chemotopic organization within the OB in fish was first indicated in salmonids (Thommesen 1978) and subsequently documented in other teleost species. Overall, but especially for catfish (Nikonov and Caprio 2001), zebrafish (Friedrich and Korsching 1998), crucian carp (Hamdani et al. 2000, 2001; Hamdani and Døving 2003; Weltzien et al. 2003), char and grayling (Døving et al. 1980), the results indicate a functional division of the OB in teleosts into lateral and medial portions (Satou 1990) subserving the processing of odor information related to feeding and social cues, respectively. Unlike terrestrial vertebrates, fish lack an accessory olfactory system; therefore, both socially related odorants (bile salts) and feeding cues (amino acids and nucleotides) are processed simultaneously in the medial and lateral OB (Nikonov and Caprio 2001). Encoding of odorant molecules into spatial patterns of activity is the first step to understanding olfactory processing, yet complex olfactory-driven behaviors in fishes such as feeding, migration, and spawning are the product of cellular activity. Chemotopic maps of odor-responsive neural space reflects networks of individual neurons processing odorant information through parallel synaptic connections. Therefore, the key to understanding odor processing lies in understanding how single neurons respond to odorant molecules and how this response influences the neural network processing a particular odorant.

An advantage for studies utilizing fish as model systems is the behavioral significance of certain odorant classes (amino acids, bile salts and nucleotides) is generally known, and the loci of activity within the OB has been defined for these molecules in several fish species (Thommesen 1978; Friedrich and Korsching 1998; Nikonov and Caprio 2001). Most single unit
investigations delving into odorant processing within the OB of teleosts focused on feeding stimuli (i.e. amino acids). Recently, a few reports were published concerning the processing of social/pheromonal stimuli by the OB (Hamdani and Døving 2003; Lastein et al. 2006). For those studies that recorded bulbar responses to bile salts, only a few stimuli were typically tested (Friedrich and Korsching 1998; Hara and Zhang 1996, 1998; Laberge and Hara 2004), limiting the ability of these studies to access details of bulbar processing about this group of socially relevant stimuli for fish. To gain a better understanding of how individual molecules are processed by single neurons, and in turn, how networks of neurons process single odorant molecules, knowledge of the molecular receptive range (MRR) of single neurons must be obtained. The concept of MRR was previously used to describe the receptive range of olfactory neurons (Imamura et al. 1992; Katoh et al. 1993; Mori and Shepherd 1994) as well as those neurons in the visual and somatosensory systems.

This chapter probes the excitatory molecular receptive range (EMRR) (Mori and Yoshihara 1995) of single neurons within the medial portions of the OB to bile salts produced by the channel catfish and structurally similar analogues. EMRR is defined as the range of odorant molecules which elicit excitatory responses from a given neuron which are likely to promote activity in downstream neurons. OB neurons respond excitedly to specific combinations of molecular features at four critical carbon positions located in the side-chain and along the steroid backbone. Based on the EMRR of the recorded neurons, three groups of bile salt responsive OB neurons were identified. The data also suggest that channel catfish can detect and discriminate those bile salts produced by conspecifics from other structurally similar bile salts.
MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Channel catfish (*Ictalurus punctatus, Rafinesque*), 15-22cm, were obtained from the Louisiana State University (LSU) aquaculture facility from both floating cages in outdoor ponds and indoor recirculating tanks. Fish were held in the LSU Animal Care Facility in a 300L aquarium filled with charcoal-filtered tap water (CFTW) and maintained on a 12h:12h light:dark regime for up to two weeks. For those fish gathered from floating cages, the water temperature was held below 20ºC during the winter months and above 31ºC during the summer months to inhibit growth of the pathogenic bacterium, *Edwardsia ictaluri*, which causes enteric septicemia and destroys chemosensory epithelia (Morrison and Plumb 1994). For those fish gathered from indoor recirculating tanks, the ambient air temperature was held at 20ºC.

ANIMAL PREPARATION

The procedures outlined below are in accordance with a protocol approved by the Institutional Animal Care and Use Committee (LSU School of Veterinary Medicine).

Each catfish was immobilized with an initial intramuscular injection of Flaxedil (gallamine triethiodide, 0.03mg/100g body weight). Subsequent injections of Flaxedil were provided as needed during experimentation via a hypodermic needle embedded in the flank musculature. After immobilization, the catfish was wrapped in wet tissue paper and secured with orbital ridge clamps in a custom-made Plexiglas® container. The gills were irrigated via a constant flow of CFTW containing the general anesthetic, MS-222, for the duration of the surgical procedures (ethyl-m-aminobenzoate methane sulfonic acid; initial concentration, 50mg/L; Sigma Chemical, St. Louis, MO). Tetracaine (3%) was applied to the surgical site 5 mins prior to surgical procedures. Minor surgery was performed to provide access to the
olfactory mucosa and olfactory bulb. Thirty minutes prior to electrophysiological recordings, the 
anesthetic-containing gill irrigation water was replaced with fresh CFTW not containing MS-222.  

STIMULUS SOLUTIONS AND DELIVERY

The odorants included L-amino acids (alanine, arginine, glutamate and methionine), bile 
salts (sodium salts of chenodeoxycholic acid [CDC], cholic acid [CA], deoxycholic acid [DCA], 
glycochenodeoxycholic acid [GCDC], glycocholic acid [GCA], lithocholic acid [LCA], 
taurochenodeoxycholic acid [TCDC], taurocholic acid [TCA], taurodeoxycholic acid [TDC], 
taurolithocholic acid [TLC] and taurocholic acid-3-sulfate [TLCS]), and nucleotides (adenosine 
5’-triphosphate [ATP], inositol 5’-monophosphate [IMP] and inositol 5’-triphosphate [ITP]).  All 
chemical stimuli were purchased from Sigma (St. Louis, MO) and were of the highest purity 
available (97%-99%).  Stock solutions of amino acids and bile salts were prepared weekly using 
CFTW and refrigerated when not in use; nucleotides were prepared monthly in CFTW and 
frozen (-20ºC) until just prior to testing.  Test solutions were diluted daily from stock solutions to 
experimental concentrations with CFTW and were tested at room temperature, the same as the 
temperature of the water flow to the olfactory organ.  The pH of each test solution, when diluted 
to experimental concentrations, matched that of the CFTW (pH~8.7).

Stimulus delivery was via a “gravity-feed” system that was previously described 
(Sveinson and Hara 2000).  Briefly, stimulus solutions and the CFTW used to bathe the olfactory 
epithelium were delivered through separate Teflon® tubes (diameter 0.8mm) to the olfactory 
mucosa at a flow rate of 5-7 ml/min.  A foot switch connected to an electronic timer (Model 645, 
GraLab Instruments Division, Dimco-Gray Corporation, Centerville, OH) triggered a pneumatic 
actuator valve to introduce the stimulus for 2s applications.  CFTW continuously perfused the 
olfactory mucosa to 1) prevent the mucosa from desiccating, 2) facilitate stimulus delivery, 3) 
avoid the introduction of mechanical artifacts associated with stimulus presentation and 4) rinse
the olfactory organ clear of any residual stimuli for a minimum of 2 mins between stimulus applications.

ELECTROPHYSIOLOGICAL RECORDING TECHNIQUES

THE ELECTROOLFACTOGRAM (EOG). The underwater EOG, a slow DC potential change in the water above the olfactory mucosa, suggested to be the summed generator potentials of the responding ORNs to odorant molecules (Ottoson 1971), was obtained in vivo with calomel electrodes via Ringer’s-agar-filled capillary pipettes (Caprio 1995). The pipette of the active electrode was positioned near the midline raphe of the olfactory organ, whereas the pipette of the reference electrode was placed against the skin adjacent to the olfactory cavity. The EOG was amplified (Grass P-18; Astro-Med Inc., West Warwick, RI), displayed on an oscilloscope and DC chart recorder, digitized and stored on a video channel of a hi-fi VCR.

SINGLE UNIT RECORDINGS FROM THE OB. Single OB unit activity was recorded extracellularly from the medial regions of the OB with low impedance (0.5-2MΩ) platinum and gold-plated, metal-filled, glass micropipettes (modified from Gesteland et al. 1959; Caprio 1995). Soda lime glass [inner diameter 1.1-1.2mm, thin wall (0.2mm)] was pulled on a vertical puller (Narishige PP-83) to provide a 2µm tip. A small rod of Cerrelow metal was inserted into the glass pipette and melted on a hot plate while being pushed with a metal rod (which also acted as a heat sink) toward the pulled tip of the glass. The electrode was electroplated for 3-10s (1.5mV battery through a 10MΩ resistor) with gold (code 3023, Sifco, Cleveland, OH) to form a 2-5µm ball followed by a Pt (5% Pt chloride) coating electroplated (5-10s through a 50MΩ resistor) over the gold. The electrode was mounted on a hydraulic microdrive and advanced vertically downward from the dorsal surface of the OB (Fig. 2.1). Recordings began once a spontaneously active unit was encountered and clearly isolated by fine-positioning of the recording electrode via the remote fluid-filled microdrive. Action potentials were amplified
(Grass Instruments P511; bandpass 30-3,000Hz), observed with an oscilloscope and stored as an analog signal on an audio channel of a hifi VCR.

DATA ANALYSIS. The recorded data were digitized at 32kHz and analyzed off-line by Discovery software (Brainwave Systems Discovery package Version 5.0 with Autocut, DataWave Technologies, Longmont, CO). Eight parameters (peak amplitude, valley amplitude, spike height, spike width, spike duration, time between spikes, ratio of peaks and ratio of areas) were utilized by the software to identify and discriminate extracellularly recorded action potentials. Spike events, EOG signals, and onset of stimulation were time-stamped with 32-bit 100µs resolution and saved in a data file. The data files were viewed and analyzed using Neuroexplorer (Nex Technologies, Lexington, MA) software.

Responses of single OB neurons were classified as excitatory, suppressive or null (not significantly different from pre-stimulus) based on the one-tailed interrupted time-series analysis (ITSA) (Crosbie 1993; Hudson 1977; Kang and Caprio 1995). The ITSA compares statistically the number of action potentials occurring within successive 250ms time bins for 2 s before and after the initial onset of the odor-induced EOG. Spike counts within the 2 s stimulus periods, which were significantly greater (P≤0.05) than spike counts 2 s immediately prior to stimulus onset, were classified as excitatory responses.

RESULTS

MOLECULAR FEATURES OF THE TESTED BILE SALT ODORANTS

The molecular features (R1-R4) of the eleven bile salts tested differed at three carbon positions, C3, C7 and C12, along the steroid backbone and at C24 of the side-chain (Fig. 2.2). The eleven bile salts tested were segregated into three major categories based on the molecular feature at C24: either (1) taurine-conjugated (TBS), (2) glycine-conjugated (GBS) or (3) non-conjugated (NBS); five of the eleven bile salts were conjugated to taurine, two to glycine and
Figure 2.1. A longitudinal section through the head of a catfish illustrating the position of the recording electrode within the OB. Modified from Cancalon (1983). m=olfactory mucosa, on=olfactory nerve, ob=olfactory bulb, ot=olfactory tract, ol=olfactory lobe (forebrain).
four were non-conjugated leaving a carboxyl moiety at C24. Within each category, hydroxylation varied at the C7 and C12 positions so that a particular bile salt species could possess hydroxyl moieties at both C7 and C12, either C7 or C12, or lack hydroxyl moieties at both positions (Fig. 2.2). Only TLCS possessed a unique R1 feature, i.e. a sulfate moiety, at C3; for all other bile salts tested, R1 was an hydroxyl moiety. All 11 bile salts were 3α, 5β, 7α, and 12α isomers.

THREE MAJOR OB NEURON GROUPS ARE EXCITED BY BILE SALTS

Fifty-one OB neurons from 30 channel catfish were recorded from the medial region of the OB. All 51 units were excited by bile salt odorants ≤10µM and not by 1-10µM L-amino acids or 10-100µM nucleotides, i.e. the other classes of biologically relevant odorants that activate different populations of OB neurons in the channel catfish (Nikonov and Caprio 2001). The majority of the recorded OB neurons (47/51; 92%) that were excited by specific bile salts tested were suppressed (i.e. a decreased spike output below that of spontaneous activity) by amino acids, nucleotides and particular bile salts. In the present study of OB neuron selectivity to bile salts, only excitatory responses [i.e. where the number of action potentials within the stimulus period (2sec) was significantly greater (P≤0.05) than that occurring spontaneously immediately prior (2sec) to stimulus application] were critically analyzed since it is the excitatory response that drives downstream neurons. However, a few findings addressing suppressive responses are reported below.

In searching for bile salt responsive OB neurons, separate mixtures composed of taurine-conjugated, non-conjugated and glycine-conjugated bile salts, respectively, were initially tested. Once an OB neuron was located that was excited by at least one of the three test mixtures, the components of that (those) mixture(s) were tested individually. In all cases studied, single neurons which responded excitedly to a particular mixture also responded excitedly to at least
Figure 2.2. The molecular formulae of the bile salts tested. Carbons of the steroid backbone and side-chain are numerically labeled (1-24). The rings of the steroid backbone are designated A-D. Molecular features, designated by R1-R4, of each bile salt tested vary at positions C3, C7 and C12 of the steroid backbone and C24 of the side-chain. The stimuli included different classes of bile salts based on the specific molecular feature (R4) attached to C24 (glycine-conjugated [GBS], taurine-conjugated [TBS], non-conjugated [NBS]). All of the bile salts above are 3α, 5β, 7α, and 12α isomers.
one component of the mixture. The 51 recorded bile salt responsive neurons were divided into 3 major groups based on their respective EMRRs. Eighteen of fifty-one (35%) neurons were classified as Group T whose EMRRs included only taurine-conjugated bile salts (Fig. 2.3A). Seventeen of fifty-one (33%) units were classified as Group N whose EMRRs included only non-conjugated bile salts (Fig. 2.3B). The remaining sixteen neurons (32%) were classified as Group G whose EMRRs included at least one bile salt from each of the three categories of bile salts chosen for this study (i.e. TBS, NBS and GBS; Fig. 2.4).

CHEMOTOPY

Neurons of each group of bile salt responsive neurons were recorded along both rostral/caudal and dorsal/ventral axes of the medial OB (Fig. 2.5) which was previously indicated to process bile salt information (Nikonov and Caprio 2001). The data do not suggest that the bile salt responsive region of the channel catfish OB is subdivided into distinct sub-regions of neurons that selectively process each of the three types of bile salts tested.

GROUP T OB NEURONS

All of the eighteen Group T neurons were excited by bile salts with particular combinations of molecular features where R4 was a taurine moiety (Fig. 2.3A); no Group T neuron responded to bile salt odorants where the molecular feature, R4, at C24 of the side-chain was a glycine moiety (glycine-conjugated) or an hydroxyl moiety (non-conjugated). Excitatory thresholds ranged from 0.1- 10µM (Table 2.1). Sixty-seven percent (12 of 18 neurons) of Group T neurons were excited by taurine-conjugated bile salts at 1µM and 11% (2 of 18 neurons) responded at 0.1µM.

Thirteen Group T neurons were excited by only one TBS (Fig. 2.3A); the remaining five neurons responded excitedly by 2-3 TBS. These neurons discriminated further taurine conjugated bile salts by molecular features (R1-3) at C3, C7 and C12 of the steroid backbone
Figure 2.3. Representative raster plots of Group T and N OB neurons. Two single unit recordings are shown: (A) a Group T neuron responding excitedly to TCDC (a taurine-conjugated bile salt) and (B) a Group N OB neuron responding excitedly to LCA (a non-conjugated bile salt). Odorants eliciting an excitatory response (P≤0.05) are marked with an asterisk. Two second odor applications are indicated by the scale bar above each set of raster plots beginning at the vertical dotted line. EOG scale bar: 0.5mV, 400ms. GBS = glycine-conjugated bile salt; TBS = taurine-conjugated bile salt; NBS = non-conjugated bile salt.
Figure 2.4. A Group G OB neuron responding excitedly to taurine-conjugated, non-conjugated and glycine-conjugated bile salts. Odorants eliciting an excitatory response are marked with an asterisk. Two second odor applications are indicated by the scale bar above each set of raster plots beginning at the vertical dotted line. EOG scale bar: 0.5mV, 400ms.
**Figure 2.5.** Group T, N and G OB neurons are distributed along both the rostral/caudal and dorsal/ventral portions of the medial region of the channel catfish OB. Stereotaxic methods were utilized to determine the location of each neuron as described previously by Nikonov and Caprio (2001). The position of each neuron depicted above is expressed as a percentage of the total length/width of the OB.
Table 2.1. OB neuron thresholds to bile salts.

<table>
<thead>
<tr>
<th>Odorant Concentration (M)</th>
<th>Number (%) of Group T units excited by TBS (n=18)</th>
<th>Number (%) of Group N units excited by NBS (n=17)</th>
<th>Number (%) of Group G units excited by GBS, NBS and TBS (n=16)</th>
<th>Totals of Groups T, N &amp; G (51 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 10⁻⁸M</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (6)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>10⁻⁷M</td>
<td>2 (11)</td>
<td>0 (0)</td>
<td>3 (19)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>10⁻⁶M</td>
<td>12 (67)</td>
<td>2 (12)</td>
<td>15 (94)</td>
<td>29 (57)</td>
</tr>
<tr>
<td>10⁻⁵M</td>
<td>18 (100)</td>
<td>17 (100)</td>
<td>16 (100)</td>
<td>51 (100)</td>
</tr>
</tbody>
</table>
Six Group T neurons were selectively excited by TLCS. TLC (which differs from TLCS by an hydroxyl group at C3), TCDC, TCA and TDC did not excite these neurons (Fig. 2.6, Neurons 1-6; Fig. 2.7A). Excitation required sulfonation at C3. The selectivity of these neurons for molecular features other than a –H at C7/C12 and taurine at C24 was not tested by this panel of bile salts (i.e. TLCS was the only C3 sulfonated bile salt tested). For neurons 7-18 (Fig. 2.6), excitatory responses required the bile salt molecule to be hydroxylated at C3 and conjugated to taurine at C24; further, these neurons discriminated individual bile salts by –OH/–H moieties located at C7 and C12. For example, four neurons were selectively excited by TCDC (Fig. 2.6, neurons 7-10; Fig. 2.7B) which possesses an –OH at C7 and a –H at C12. In contrast, neurons 11 and 12 responded excitedly to only TCA which possesses –OH groups at both C7 and C12. Neurons 14-17 were slightly less selective responding excitedly to both TCDC and TCA where both molecules possess similar molecular features at C3, C7 and C24 (Fig. 2.6). As a whole, the EMRRs of Group T neurons included multiple molecular features along the steroid backbone (R1-R3) and the side-chain (R4) of taurine-conjugated bile salts.

GROUP N OB NEURONS

Group N neurons were excited by only NBS having an hydroxyl moiety (R4) at C24 in the side-chain. Excitatory thresholds for Group N OB neurons ranged from 1µM-10µM. All Group N neurons responded excitedly to non-conjugated bile salts at 10µM while 12% (2 of 17 neurons) were excited at 1µM (Table 2.1). These neurons, like those of Group T, discriminated further molecular features (R2 and R3) along the steroid backbone at C7 and C12. Eleven of seventeen (65%) Group N neurons responded to only one NBS; the remaining six neurons (35%) responded to 2-3 non-conjugated bile salts (Fig. 2.8). Eight Group N neurons were selectively excited by LCA (Fig. 2.8 Neurons 1-8; Fig. 2.9A). For these neurons, the EMRR included three molecular features: R2= a –H at C7, R3= a –H at C12 and R4= an –OH at C24. Neurons 12-17
Figure 2.6. Group T OB neurons are excited by only TBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the OB neuron activity to the odorant did not change from pre-stimulus levels. The molecular features determined to be critical for eliciting excitatory responses are indicated above, excluding R4, which was a taurine moiety.
Figure 2.7. Representative Group T OB neurons. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. (A) A Group T neuron (Neuron 5 in Fig. 2.6) solely activated by TLCS (I). TLC (II), which differs from TLCS only by a –OH at R3 did not activate this neuron. Two second odor applications indicated by the black horizontal line in each rate histogram. (B) A single OB neuron (Neuron 7 in Fig. 2.6) excited by TCDC (I) and unresponsive to TCA (II), TDC (III), TLC (IV) and TLCS (rate histogram not shown). Ordinate scales for all rate histograms in BII-IV are the same as BI; note the differences in scaling of the y-axis in A and B. Each rate histogram was smoothed with a Gaussian filter.
responded to 2-3 NBS (Fig. 2.8). For these neurons, the molecular features required for excitatory responses were relaxed at the C7 and C12 positions; however, clear preferences for combinations of molecular features are evident. For example, eliciting excitatory responses from neurons 13 and 14 required a −H at C12 irrespective of the molecular feature at C7 (Figs. 2.8, 2.9B); DCA and CA, both of which possess −OH moiety at C12, did not excite these neurons.

As a whole, the EMRR of Group N OB neurons included molecular features at R2, R3 and R4. The selectivity of Group N neurons for the molecular feature at R1 could not be determined by the panel of bile salts tested in this study as R1 was a hydroxyl moiety in all 4 NBS tested.

GROUP G OB NEURONS

All sixteen OB neurons classified as Group G responded excitedly to at least one glycine-conjugated, one taurine-conjugated and one non-conjugated bile salt (Fig. 2.10). Excitatory thresholds for Group G neurons ranged from 0.1µM to 10µM. Ninety-four percent (15 of 16 neurons) of Group G neurons were excited by at least one of the 11 bile salts tested at 1µM while 19% (3 of 16 neurons) responded at 0.1µM (Table 2.1). Only neuron 16 (Fig. 2.10) responded excitedly to a bile salt at concentration ≤0.01 µM. For this neuron, excitatory responses were recorded to 0.1nM-10µM CA. Group G neurons responded particularly well to GBS. For Group G neurons, only GCDC elicited an excitatory response from all sixteen neurons within this group, while 81% (13 of 16 neurons) were also excited by GCA. Furthermore, Group G neurons responded well to bile salts with a hydroxyl group as the molecular feature at C7 regardless of the molecular feature at C24. Eighty-eight percent (14 of 16 neurons) of Group G neurons were activated by TCA or TCDC, and all 16 Group G neurons were excited by either CA or CDC. All four of these bile salts possess a hydroxyl moiety at C7. Conversely, the two taurine-conjugated (TLC, TLCS) and two non-conjugated (LCA and DCA) bile salts lacking a
Figure 2.8. Group N OB neurons are excited by only NBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the OB neuron activity to the odorant did not change significantly from pre-stimulus levels. The molecular features determined to be critical for eliciting an excitatory response are indicated above, excluding R4, which was an hydroxyl moiety.
Figure 2.9. Representative Group N OB neurons. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. Two separate OB neurons are shown in A and B. (A) An OB neuron excited by LCA (I). Rate histograms for neuron activity in response to CDC (II), DCA (III) and CA (IV) are also shown to allow for comparison. Excitatory responses in this neuron required two molecular features along the steroid backbone (R2 = –H and R3 = –H) and one in the side-chain, R4 = –OH. (B) A Group N OB neuron activated by both LCA (I) and CDC (II). Rate histograms for neuron activity in response to DCA (III) and CA (IV) are also shown to allow for comparison. Excitatory responses in this neuron required one molecular feature of the steroid backbone (R3 = –H) and one of the side-chain (R4 = –OH). Two-second odor applications indicated by the black horizontal line in each rate histogram. Ordinate scales for the rate histograms are indicated in AI and BI; note the differences in scaling of the y-axis in A and B. Each rate histogram was smoothed with a Gaussian filter.
hydroxyl group at C7 (R2=–H for these bile salts) elicited excitatory responses from only 19% (3 of 16 neurons) and 13% (2 of 16 neurons) of the Group G neurons tested, respectively. The lone exception to this trend was TDC (R2 = –H) to which 56% (9 of 16 neurons) of Group G neurons responded excitedly (Fig. 2.10). The EMRRs of Group G neurons were the least complex of Groups T, N and G, including most bile salts which possess hydroxyl moieties at both C3 and C7.

SUPPRESSIVE RESPONSES

Thirty-six of the fifty-one recorded OB neurons (71%) were suppressed by ≥ 3 of the eleven tested bile salts. Often the bile salt odorants eliciting suppressive responses were structurally similar to the bile salt odorants eliciting excitatory responses; however, this was not always the case. For example, notice that TLC (R1= -OH) suppressed the spontaneous activity of neurons excited by TLCS [(R1=SO₄); Fig. 2.6, Neurons 1-6, Fig. 2.7A]. TLC (R2=H, R3=H, R4=NHCH₂CH₂SO₃H) also suppressed the spontaneous activity of neurons excited by LCA [(R2=H, R3=H and R4=OH); Fig. 2.8, neurons 1-8]. Further, the spontaneous activity of neurons excited by LCA were generally suppressed by other non-conjugated bile salt odorants (Fig. 2.8; neurons 1-8). We reasoned that the dynamic response capacity (i.e. the ability to increase and decrease spike output during odorant stimulation) of bile salt-responsive neurons allows for a greater contrast enhancement of the output of the OB network, sharpening the combinatorial input received by downstream targets.

DISCUSSION

BILE SALTS IN FISHES AND THEIR FUNCTION AS OLFACTORY CUES

Investigations involving salmonids and sea lamprey currently provide the best evidence as to the role of bile salts in fish olfaction. Anadromous fishes utilize olfactory cues to locate suitable spawning areas within streams and tributaries with successive generations returning to
Figure 2.10. Group G OB neurons are excited by TBS, GBS and CBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the OB neuron activity to the odorant did not change significantly from pre-stimulus levels. For unit 16 only, excitatory responses were recorded to 0.1nM-10µM CA.
the area where they were spawned (Hasler and Scholz 1983; Smith 1985; Stabell 1992). Nordeng (1971, 1977) proposed that homeward migration is an inherited response to this population-specific odor learned prior to downstream migration and termed this the pheromone hypothesis. Both juvenile salmonids that begin migration to the sea and those which remain within the specific stream where they hatched provide a continual source of population-specific odor in the water system to which adults orient and respond. Based on electrophysiological data, bile salts were later suggested to be a component of this population-specific odor (Døving et al. 1980). Presently, however, no direct behavioral evidence was reported which indicates that bile salts mediate the homeward migration of salmonids. However, reports do exist suggesting that specific bile salts emanating from sea lamprey (*Petromyzon marinus*) mediate migration in this species (Bjerselius et al. 2000; Fine et al. 2004; Fine and Sorensen 2005; Li et al. 1995; Li and Sorensen 1997; Li et al. 2002; Polkinghorne et al. 2001; Vrieze and Sorensen 2001). It is hypothesized that recognition and discrimination of specific sea lamprey bile salts from those of other species is key for a successful migration of sea lamprey to suitable spawning habitats.

Sexually mature sea lampreys innately recognize a mixture of species-specific bile salts (Li et al. 1995; Li et al. 2002; Fine et al. 2004; Sorensen et al. 2005) and select for streams containing populations of sea lamprey larvae which would indicate suitable spawning grounds (Bjerselius et al. 2000; Polkinghorne et al. 2001; Vrieze and Sorensen 2001; Fine and Sorensen 2005).

Electrophysiological data indicate that sea lampreys possess a complement of molecular olfactory receptors (mORs) allowing for the detection and discrimination of conspecifics bile salts, produced by adults or larvae (Li et al. 1995). These studies utilized electrophysiological cross-adaptation experiments which suggest that mORs bind particular combinations of molecular features (-SO₄, -OH and conjugating moieties) located at C24 of the side-chain and C7/C12 of the steroid nucleus of bile salts allowing for the differentiation of structurally similar
bile salt molecules. However, it is unknown in sea lamprey as to how bile salt information is processed by higher olfactory centers (i.e. the olfactory bulb and forebrain).

In teleosts, behavioral studies demonstrated that freshwater eels (Sola and Tosi 1993), cod (Hellstrom and Døving 1986) and Artic char (Jones and Hara 1985) respond to synthetic bile salts with activities classified as orientation and snapping. Previous electrophysiological investigations utilizing EOG and multi-unit recordings confirmed the stimulatory effectiveness of bile salts to either the olfactory epithelium or the olfactory bulb of char and graylings (Thommesen 1978; Døving et al. 1980; Zhang et al. 2001), trout (Hara et al. 1984; Laberge and Hara 2004), salmon (Hara and Zhang 1996), zebrafish (Michel and Lubomudrov 1995; Michel and Derbidge 1997; Friedrich and Korsching 1998) and channel catfish (Nikonov and Caprio 2001).

The studies discussed above indicate that bile salts activate the initial portion of the olfactory system and describe the resulting behavior. However, few studies investigated the molecular aspects by which the teleost olfactory system possibly discriminates bile salt molecules. The findings of the current study indicate that OB neurons of the channel catfish respond selectively to specific combinations of molecular features present on the side-chain and steroid nucleus of the bile salt molecule. Moreover, the EMRR of OB neurons is comprised of specific combinations of molecular features present on the side chain and steroid nucleus of bile salts. The present report provides evidence for how OB neurons process bile salt odorant information which allows for the extrapolation of the EMRRs of channel catfish ORNs that provide input to the OB.
FISH Olfactory Systems Respond to the Molecular Features of Bile Salt Molecules

The present report indicates that OB neurons of the channel catfish are excited by specific molecular features present at four carbon positions, C3, C7, C12 and C24 of bile salt molecules. We categorized these OB neurons based on their EMRR for the molecular feature (R4) at C24, allowing for the identification of 3 major groups of bile salt responsive OB neurons (Groups T, N and G). Group T and N neurons were excited exclusively by TBS and NBS, respectively, while Group G neurons were excited by at least one TBS, one GBS and one NBS. A previous electrophysiological cross-adaptation study (Michel and Derbidge 1997) suggested that zebrafish possess mORs capable of discriminating taurine-conjugated, glycine-conjugated and non-conjugated bile salts, highlighting the importance of the conjugating group (R4). This previous study did not address the putative role of –OH/–H moieties at C7 and C12. However, significant non-reciprocal cross-adaptation occurred for two bile salt pairs (Michel and Derbidge 1997). GCDC significantly adapted EOG responses to GCA, and TCDC significantly adapted EOG responses to TCA although the reverse was not observed statistically. These data suggest that GCDC/GCA and TCDC/TCA share a significant number of mOR sites. Therefore, it is likely that a large portion of the information regarding to these pairs of bile is transmitted along the same neural pathways (OB and forebrain) in zebrafish. The present study found both (1) OB neurons with EMRRs which included both GCDC and GCA or TCDC and TCA and (2) OB neurons capable of discriminating these pairs of bile salts. Given that ORNs with like mORs converge onto the same glomerulus in the OB, we reasoned that the EMRR of OB neurons projecting dendrites to these glomeruli would greatly reflect the EMRR of the afferent ORNs, thus allowing a comparison of EMRRs of first order and second order olfactory sensory neurons. In light of the current study in channel catfish, the non-reciprocal cross-adaptation observed in
zebrafish may reflect the olfactory organ processing of the molecular feature at C12. These data suggest that GCDC/GCA and TCDC/TCA odorant information is transmitted along similar olfactory pathways in fish with moderate overlap; however, some neurons remained responsive (i.e. excited) to one, but not the other which suggests that both zebrafish and channel catfish perceive these bile salt pairs as different odorants. It is interesting to note that rainbow trout discriminate behaviorally TCDC and TCA in conditioning trials (Thwaits et al. 2006).

In the present study, OB neurons selective for TLCS were recorded and categorized as Group T. Lake char (Zhang and Hara 1994) and sea lamprey (Li and Sorensen 1997; Siefkas and Li 2004) were also reported to possess independent mOR sites for sulfonated bile salts. Li and Sorensen (1997) suggested that TLCS, LCS (lithocholic acid-3-sulfate) and GLCS (glycolithocholic acid-3-sulfate) likely bind to a common mOR selective for a sulfate moiety at C3 regardless of the conjugating group at C24. The categorization of TLCS responsive OB neurons in the present study may need to be modified in the future when more C3 sulfonated bile salts with variable R4 molecular features are tested. It is likely that the TLCS responsive OB neurons reported here are a group separate from those that are selective for taurine-conjugated bile salts possessing an hydroxyl group at C3.

**SINGLE OB NEURONS SELECTIVITY DETERMINED ELECTROPHYSIOLOGICALLY VS GROSS OB IMAGING**

Previous experiments investigated the spatial arrangement of ORN input to the zebrafish OB by visualizing the activity patterns evoked by individual bile salts, both glycine-conjugated and taurine-conjugated, with voltage-sensitive dyes loaded into ORNs (Friedrich and Korschning 1998). Patterns of bile salt evoked activity in zebrafish occurred predominately in the anterior medial portions of the OB. The activity patterns elicited by GCA and TDC included specific OB regions that were activated by only one of the compounds (specific areas) and other areas that
were co-activated by both bile salts (non-specific). Odorant evoked activity patterns for GCA and TDC in zebrafish partially overlapped within the ventro-medial OB. In the present study in catfish, OB neurons were identified that were excited by TBS only (Group T) and those excited by TBS and GBS (Group G). Further, neurons of both Group G and Group T were intermingled with one another within the medial region of the catfish OB. Intermingling of OB neurons possessing EMRRs for both GCA/TDC (i.e. Group G neurons) and those neurons having EMRRs for just one of the two bile salts, as seen in channel catfish, would produce the types of activity patterns seen in zebrafish.

THE EMRRs OF CATFISH OB NEURONS VS THE EMRR OF THE HUMAN BILE SALT RECEPTOR

The OB neurons recorded in the present study exhibited selectivity for particular molecular features (R1-R4) of the bile salt molecule. Since, the EMRR of a bile salt mOR has not yet been characterized no direct comparison between our electrophysiological results and mOR specificity can presently be made. Recently, however, a human plasma membrane bile salt receptor (TGR5/BG37) was identified and its selectivity to a panel of structurally diverse bile salts was determined (Maruyama et al. 2002; Kawamata et al. 2003). TGR5/BG37 and fish mORs are structurally similar (7-transmembrane domain) and both TGR5/BG37 (Maruyama et al. 2002; Kawamata et al. 2003) and fish mORs (Hansen et al. 2003) couple to the cAMP signaling cascade. Maruyama et al. (2002) and Kawamata et al. (2003) reached similar conclusions as to the specificity of TGR5/BG37 for bile salts: (1) the receptor is most strongly activated by bile salts lacking hydroxyl groups at C7 and C12, addition of hydroxyl groups to one or both locations results in less cAMP production, and (2) taurine-conjugation, glycine-conjugation nor non-conjugation played a minor role in receptor activation. These data demonstrate the importance of the molecular features at C7 and C12 for TGR5/BG37-ligand
interactions. In the present study, the molecular features present at C7 and C12 were highly important in determining the EMRR of neurons within Groups T, N and G. Further, the molecular feature at C24 appears to be more important for determining the EMRR of neurons in Groups T and N as compared to Group G.
CHAPTER 3

PROCESSING OF BILE SALT ODOR INFORMATION BY SINGLE FOREBRAIN NEURONS IN THE CHANNEL CATFISH
INTRODUCTION

The basic organization of the olfactory system is conserved across vertebrates (Ache and Young 2005). Axons of ORNs expressing the same odorant receptor protein converge onto glomeruli in the OB forming synapses with the apical dendrites of mitral/tufted cells. The spatial arrangement of these glomeruli form a chemotopic map across the surface of the OB relating general chemical features of odorant molecules to specific glomerular fields (Xu et al. 2000). Thus, each odorant activates specific patterns of glomerular activity unique to each odorant molecule. Further, recent information indicates, at least for the channel catfish, a chemotopic map grossly similar to that of the OB extends across the olfactory nuclei of the forebrain (Nikonov et al. 2005). To better understand how the CNS processes bile salt odorant information, knowledge of the molecular receptive range (Mori and Yoshihara 1995) of single neurons is required at this neuronal level.

The data presented in this chapter investigates the EMRR of single neurons within the medial portions of the FB to bile salts produced by the channel catfish and structurally similar analogues. These areas receive synaptic input from mitral cells lying within the medial portions of the olfactory bulb (Bass 1981; Finger 1975), which were the focus of the previous chapter. FB neurons were divided into three main categories based on the molecular features of bile salt molecules that fall within the EMRR of each neuron. The EMRRs of bile salt responsive FB neurons of the channel catfish include multiple features present on a given bile salt molecule. When comparing data from the present chapter to those of chapter 2, the EMRRs of bile salt responsive neurons in both the OB and FB are highly similar in this species.

MATERIALS AND METHODS

The materials and methods utilized in the present study were identical to those reported previously (Chapter two) with the following exceptions: 1) minor surgery was performed to
provide access to the olfactory mucosae and the FB, 2) one second stimulus applications were applied simultaneously to both olfactory epithelia, 3) low impedance (0.5-1MΩ) microelectrodes were used for all FB recordings and 4) response type determination (described below).

Single FB neurons responses were recorded from both the right and left cerebral lobes. The electrode position is illustrated in (Fig. 3.1).

Responses of single FB neurons were classified as excitatory, suppressive or null (not significantly different from pre-stimulus) based on the one-tailed interrupted time-series analysis (ITSA) (Crosbie 1993; Hudson 1977; Kang and Caprio 1995). The ITSA compares statistically the number of action potentials occurring within successive 200ms time bins for 1 sec before and after the initial onset of the odor-induced EOG. Spike counts within the 1 sec stimulus periods, which were significantly greater (P≤0.05) than spike counts 1 sec immediately prior to stimulus onset, were classified as excitatory responses.

RESULTS

The present study investigated the responses of 68 FB neurons obtained from 17 channel catfish to determine their EMRRs for eleven tested bile salts. Neurons from each group were recorded from the medial regions of the FB receiving synaptic input from the medial olfactory tract (Bass 1981; Finger 1975; Fig. 3.2) at depths of 1,000µm-1,800µm. Forty-five neurons responded excitedly to at least one bile salt odorant, but all sixty-eight were not excited by 1-10µM L-amino acids or 1-10µM nucleotides. Only excitatory responses were critically analyzed in the present study because it is the excitatory response that drives excitatory responses in downstream neurons. However, an additional 23 single FB neurons were recorded that responded to bile salt odorants with suppressive responses only (n=14) or resulted in no significant change in spontaneous activity (n=9, Table 3.1).
Figure 3.1. A longitudinal section through the head of a catfish illustrating the position of the recording electrode within the FB. Modified from Cancalon (1983). m=olfactory mucosa, on=olfactory nerve, ob=olfactory bulb, ot=olfactory tract, ol=olfactory lobe (forebrain).
Figure 3.2. Group T, N and G neurons were recorded in the ventro-medial regions of the channel catfish FB. Stereotaxic methods were utilized to determine the location of each neuron as described previously by Nikonov et al. 2005. The position of each neuron depicted above is expressed as a percentage of the total length/width of the FB.
FB neurons categorized into the latter two groups were tallied only if: 1) the spike trains were recorded at an electrode location simultaneously with the spike train of a FB neuron responding excitedly to bile salt stimuli, 2) the activity persisted above background noise throughout the recording period, and 3) the entire stimulus panel was tested.

Table 3.1. In response to bile salt stimulation, activity of FB neurons was classified as excitation, suppression or null.

<table>
<thead>
<tr>
<th>Number of FB neurons recorded</th>
<th>FB neurons responding excitedly to bile salts</th>
<th>FB neurons responding to bile salts with only inhibitory responses</th>
<th>FB neurons lacking responses to all of the tested odorants (BS, AA, Nuc)</th>
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</thead>
<tbody>
<tr>
<td>45</td>
<td>14</td>
<td>9</td>
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</table>

In searching for bile salt responsive FB neurons, separate mixtures composed of taurine-conjugated, non-conjugated and glycine-conjugated bile salts, respectively, were initially tested. Once a FB neuron was located that was excited by at least one of the three test mixtures, the components of each mixture were tested individually. In all cases, neurons that responded excitedly to a particular mixture also responded excitedly to at least one component of the mixture. The 45 FB neurons responding excitedly to bile salts were initially divided into three major categories based on their respective EMRRs to the conjugating group (C24) of the bile salt molecule (Fig. 3.3). Seventeen of forty-five (38%) neurons were classified as Group T whose EMRRs included only TBS (Fig. 3.4A). Thirteen of forty-five (29%) neurons were classified as Group N whose EMRRs included only NBS (Fig. 3.4B). The remaining fifteen (33%) neurons were classified as Group G whose EMRRs included at least one bile salt from each of the three categories of bile salts tested (TBS, NBS and GBS; Fig. 3.5).
GROUP T FB NEURONS

Group T FB neurons were selectively excited by TBS that are conjugated to taurine at C24 and not excited by NBS or GBS. Excitatory thresholds ranged from 0.01-10µM (Table 3.2). Nine of seventeen (53%) Group T neurons were excited at 0.01µM, fifteen of seventeen (88%) neurons were excited at 0.1µM and all seventeen neurons were excited by 1-10µM TBS.

Eleven Group T neurons were excited by only one TBS (Neurons 1-11, Fig. 3.6); the remaining six neurons (Neurons 12-17) responded excitedly to 2 or 4 TBS. The data indicate that Group T neurons further discriminate TBS by the molecular features, R1-3, present at C3, C7 and C12. For neurons 1-11, excitatory responses were obtained only if critical molecular features were present at three specific locations (R1, R2 and R3). The EMRRs of neurons 1-5 (Fig. 3.6) included R1=OH, R2=OH and R3=H. TCA, TDC, TLC or TLCS did not excite these neurons since each of these bile salts possesses a different combination of -SO$_4$, –OH and –H moieties at R1, R2 and R3 (Fig. 3.3). The selectivity of neuron 1 (Fig. 3.6) can be seen in the rate histograms for TCDC, TCA, TDC and TLC (Fig. 3.7). The EMRRs of neurons 6 and 7 included R1=OH, R2=OH and R3=OH. TDC and TCDC, lacking a single –OH moiety at R2 and R3, respectively, did not excite these neurons. The EMRRs of neurons 8-11 included R1=SO$_4$, R2=H and R3=H. TLC, which differs from TLCS by a singular molecular feature, R1=OH, did not excite these neurons.

Five Group T neurons (12-16; Fig. 3.6) were excited by two TBS. Excitatory responses were obtained from these neurons only if critical molecular features were present in at least two locations. For example, the EMRRs of neurons 12-14 included R1=OH and R2=OH. These three neurons did not discriminate well TCDC and TCA whose molecular feature at R3 is an –H and –OH moiety, respectively. The EMRR of neuron 17 was the only recorded Group T EMRR
Figure 3.3. The molecular formulae of the bile salts tested. Carbons of the steroid backbone and side-chain are numerically labeled (1-24). Molecular features, designated by R1-R4, of each bile salt tested vary at positions C3, C7 and C12 of the steroid backbone and C24 of the side-chain. The rings of the steroid backbone are designated A-D. The stimuli included different classes of bile salts based on the specific molecular feature (R4) attached to C24 (glycine-conjugated [GBS], taurine-conjugated [TBS], non-conjugated [NBS]). All of the bile salts above are 3α, 5β, 7α, and 12α isomers.
Figure 3.4. Representative raster plots of Group T and N FB neurons. Two single unit recordings are shown: (A) a Group T FB neuron (Neuron #1 in Fig. 3.6) responding excitedly to TCDC (a taurine-conjugated bile salt) and (B) a Group N FB neuron (Neuron #2 in Fig. 3.8) responding excitedly to LCA (a non-conjugated bile salt). Odorants eliciting an excitatory response (P≤0.05) are marked with an asterisk. One-second odor applications are indicated by the scale bar above each set of raster plots beginning at the vertical line. EOG scale bar: 0.5mV, 500ms. GBS = glycine-conjugated bile salt; TBS = taurine-conjugated bile salt; NBS = non-conjugated bile salt.
**Figure 3.5.** A Group G FB neuron (Neuron #1 in Fig. 3.10) responding excitedly to taurine-conjugated, non-conjugated and glycine-conjugated bile salts. Odorants eliciting an excitatory response are marked with an asterisk. One-second odor applications are indicated by the scale bar above each set of raster plots beginning at the vertical line. EOG scale bar: 0.5mV, 500ms.
Table 3.2. FB neuron thresholds to bile salts.

<table>
<thead>
<tr>
<th>Odorant Concentration (M)</th>
<th>Number (%) of Group T units excited by TBS (n=17)</th>
<th>Number (%) of Group N units excited by NBS (n=13)</th>
<th>Number (%) of Group G units excited by GBS, NBS and TBS (n=15)</th>
<th>Totals of Groups T, N &amp; G (45 cells)</th>
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<tbody>
<tr>
<td>10^{-8}M</td>
<td>9 (53)</td>
<td>1 (8)</td>
<td>9 (60)</td>
<td>19 (42)</td>
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<tr>
<td>10^{-7}M</td>
<td>15 (88)</td>
<td>10 (77)</td>
<td>15 (100)</td>
<td>40 (89)</td>
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<tr>
<td>10^{-6} - 10^{-5}M</td>
<td>17 (100)</td>
<td>13 (100)</td>
<td>15 (100)</td>
<td>45 (100)</td>
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Figure 3.6. Group T FB neurons are excited by only TBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the FB neuron activity to the odorant did not change (P>0.05) from pre-stimulus levels.
Figure 3.7. A representative Group T FB neuron. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. A single FB neuron (Neuron #1 in Fig. 3.6) excited by TCDC (I) and unresponsive to TCA (II), TDC (III), TLC (IV). Excitatory responses in this neuron required two molecular features along the steroid backbone (R2 = –OH and R3 = –H) and one in the side-chain, R4 = taurine moiety. Each rate histogram was smoothed with a Gaussian filter. One-second odor applications indicated by the black horizontal line in each rate histogram.
to include <3 specific molecular features; the molecular features required to activate this neuron were R1=\(-\text{OH}\) and R4= a taurine moiety.

GROUP N FB NEURONS

Group N FB neurons were selectively excited by NBS that possess an \(-\text{OH}\) at C24 but were not excited by TBS or GBS. Excitatory thresholds ranged from 0.01-10\(\mu\)M (Table 3.2). One of thirteen (8%) Group N neurons were excited at 0.01\(\mu\)M, ten of thirteen (77%) neurons were excited at 0.1\(\mu\)M and all 13 neurons were excited by 1-10\(\mu\)M NBS.

Nine Group N neurons (Neurons 1-9) were excited by only one NBS (Fig. 3.8); the remaining four neurons were excitedly by 2-3 NBS. Like Group T neurons, Group N neurons further discriminated NBS by the molecular features (R2-3) present at C7 and C12. All four NBS tested possessed an \(-\text{OH}\) moiety at R=1 which did not allow the present study to determine the EMRRs of Group N neurons for this molecular feature. For neurons 1-9, excitatory responses were obtained to specific NBS (R4=\(-\text{OH}\) in all NBS) only if critical molecular features were present at two specific locations (R2 and R3). The EMRRs of neurons 1-5 (Fig. 3.8) included R2=H and R3=H. Neither, CA, CDC or DCA elicited excitatory responses from these neurons; each of these bile salts possesses a different combination of \(-\text{OH}\) and \(-\text{H}\) moieties at R2 and R3 (Fig. 3.3). The selectivity of neuron 1 (Fig. 3.8) can be seen in the rate histograms for LCA, CDC, DCA and CA (Fig. 3.9). The EMRR of neuron 6 included R2=\text{OH} \text{ and } R3=H.

LCA and DCA, lacking \(-\text{OH}\) moieties at R2, did not elicit excitatory responses from this neuron. The EMRRs of neurons 7-9 included R2=\text{OH} \text{ and } R3=\text{OH}. CDC, LCA and DCA, which possess a different combination of molecular features at R2 and R3, did not elicit excitatory responses from these neurons. Four Group N neurons (Neurons 10-13; Fig. 3.8) were excited by 2-3 NBS. For neurons 10 and 11, excitatory responses were obtained from these neurons only if a single \(-\text{OH}\) moiety was present at R2 or R3. CA, which possesses \(-\text{OH}\) moieties at both R2 and
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- **10^{-8}M**
- **10^{-7}M**
- **10^{-6} - 10^{-5}M**
- **Suppression**

**Figure 3.8.** Group N FB neurons are excited by only NBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the FB neuron activity to the odorant did not change significantly (P>0.05) from pre-stimulus levels.
R3 did not elicit excitatory responses from these neurons. The EMRRs of neurons 12 and 13 included R2=−OH. Both of these neurons did not discriminate the molecular feature at R3.

GROUP G FB NEURONS

All fifteen FB neurons classified as Group G responded excitedly to at least one GBS, one TBS and one NBS (Fig. 3.10). Excitatory thresholds for Group G neurons ranged from 0.01µM to 0.1µM. Nine of fifteen (60%) Group G neurons were excited by at least one of the 11 bile salts tested at 0.01µM while all 15 neurons responded at 0.1µM (Table 3.2). Group G neurons responded particularly well to GBS. Only GCDC elicited an excitatory response from all fifteen Group G neurons, while eleven of fifteen (73%) were also excited by GCA. Furthermore, Group G neurons responded well to bile salts with a hydroxyl group as the molecular feature at C7 regardless of the molecular feature at C24. Twelve of fifteen (80%) Group G neurons were excited by either TCA or TCDC, while either CA or CDC but not always both, elicited an excitatory response from every Group G FB neuron. All four of these bile salts possess a hydroxyl moiety at C7. Conversely, TLC and two non-conjugated (LCA and DCA) bile salts lacking a hydroxyl group at C7 (R2=−H for these bile salts) elicited excitatory responses from only five of fifteen Group G neurons (33%) tested. TDC (R2=−H) was moderately stimulatory for Group G neurons activating seven of fifteen (47%) group G neurons (Fig. 3.10). The EMRRs of Group G neurons were the least complex of Groups T, N and G, and included most bile salts that possess hydroxyl moieties at both C3 and C7.

DISCUSSION

THE CHEMOTOPIC MAP IN FISH: SOCIAL STIMULI PROCESSED MEDIALLY, FEEDING STIMULI PROCESSED LATERALLY

Recent evidence in teleosts suggests that social stimuli and feeding cues are processed within the medial and lateral olfactory system, respectively. This functional distinction was
Figure 3.9. A representative Group N FB neuron. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. A single FB neuron (Neuron #1 in Fig. 3.8) excited by LCA (I). Rate histograms for neuron activity in response to CDC (II), DCA (III) and CA (IV) are also shown to allow for comparison. Excitatory responses in this neuron required two molecular features along the steroid backbone (R2 = –H and R3 = –H) and one in the side-chain, R4 = –OH. One-second odor applications indicated by the black horizontal line in each rate histogram. Each rate histogram was smoothed with a Gaussian filter.
Figure 3.10. Group G FB neurons are excited by TBS, GBS and NBS. Filled circles indicate excitatory responses. Open circles indicate inhibitory responses. No symbol indicates the FB neuron activity to the odorant did not change significantly (P>0.05) from pre-stimulus levels.
demonstrated by investigations describing spatial maps of odor information in the OB of char (Thommesen 1978), zebrafish (Friedrich and Korsching 1998), channel catfish (Nikonov and Caprio 2001) and crucian carp (Hamdani et al. 2001, 2003). Further, the axons of mitral cells lying within the medial and lateral OB form the medial and lateral olfactory tracts, respectively, maintaining this functional division (Satou 1990; Hamdani 2001, 2003) for the transmission of odor information to the FB. A previous study in channel catfish reported the presence of a chemotopic map of odor information beyond the OB in the FB (Nikonov and Caprio 2005) indicating that within the FB, as was found for the OB, feeding stimuli are processed laterally and social stimuli are processed medially. Thus, for teleosts, vertebrates lacking an accessory olfactory system, spatial maps are a schematic of confined neuronal activity processing behaviorally distinct classes of odorants. Spatial maps reveal the loci of activity of neurons responding to biologically relevant stimuli, but to understand how the neural circuits within these spatial maps process odor information requires knowledge of the MRR of single neurons within these regions. The goal of the present study is to examine the EMRRs of single bile salt-responsive neurons lying within the medial FB allowing for the determination of the important molecular features of bile salt molecules which drive excitatory activity in FB neural circuits. This would also allow for a comparison of these data with those from a previous investigation of the EMRRs of OB neurons (chapter 2; Rolen and Caprio 2007).

**BILE SALTS AND THEIR BEHAVIORAL SIGNIFICANCE IN FISH**

Bile salts are commonly utilized as a representative class of odorant to investigate how social stimuli are processed by the olfactory system of fish (Hara 1984; Doving et al. 1980; Friedrich and Korschling 1998; Nikonov et al. 2005). Bile salts are biliary steroids synthesized by the liver, stored in the gall bladder and released into the intestinal lumen. Although most bile salts are reabsorbed by the enterohepatic system, in fishes some are released into the water
column in feces and urine that could possibly function as odorant molecules. The behavioral significance of bile salts was demonstrated for salmonids and sea lamprey (Jones and Hara 1985; Li et al. 2002; Sorensen et al. 2005; also see the last subsection of this chapter); however, the behavioral significance for the olfactory detection of bile salts in catfish is currently unknown. Two lines of evidence suggest the importance of this class of odorants for catfish: 1) one third of the chemotopic map in both the OB and FB is dedicated to the processing of bile salt odorant stimuli, and 2) the EMRRs of single OB neurons are tightly tuned to the molecular features of bile salt molecules tested (chapter 2; Rolen and Caprio 2007).

**BILE SALTS ARE PROCESSED IN THE MEDIAL FB, ARE OTHER SOCIAL STIMULI?**

All 68 FB neurons reported in the present study were recorded from the medial regions of the channel catfish FB that corresponds to the terminal fields of the medial olfactory tracts (areas Vd and Vv, Bass 1981; mtf, Finger 1975), FB regions that were previously indicated to process bile salt odorants (Nikonov et al. 2005). The recording session began once a single neuron (target neuron) responding excitedly to one of the bile salt mixtures was located and the electrode position was adjusted so that the action potentials of this neuron were at least 3-5 times as large as background noise. In the majority of the recordings sessions, activity from other neurons (besides the target neuron’s activity) were clearly visible throughout the recording session and could later be grouped into distinct clusters by the spike analysis software.

Typically, one to three neurons were recorded at any given electrode location. Single FB neurons were recorded which responded to bile salt stimuli with excitation (n=45), suppression only (n=14), or were unresponsive (n=9; i.e. application of bile salt stimuli did not significantly change the number of action potentials produced during the stimulus period). These data suggest that the bile salts chosen for the present study were adequately diverse to activate the majority of neurons within the bile salt responsive region of the channel catfish forebrain. Forty-five (76%)
of the fifty-nine recorded neurons whose spontaneous activity was significantly changed by application of bile salt stimuli, responded with excitation. Although the focus of the present report is to describe the EMRR of FB neurons, a few points addressing the FB neurons that did not respond excitedly to bile salts are worthy of note and are discussed below. Fourteen (24%) of sixty-eight FB neurons recorded responded to bile salt stimuli with inhibitory responses only indicating that the stimulus panel did not include bile salts possessing the necessary molecular features to activate these neurons. Given the diverse nature of bile salt molecules, this was not unexpected. The bile salts utilized in the present study included three produced by the channel catfish (TCDC, TCA and TDC; Kellogg 1975) and other structurally similar bile salts produced by other fish species. Teleosts, the largest group of extant vertebrates, synthesize a vast array of structurally diverse bile salt molecules: cyprinol sulfate, chimaerol, cholic acid, chenodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid (Denton et al. 1974; Tammer 1974; Zhang et al. 2001; Yeh and Hwang 2001; Thwaits et al. 2006). Therefore, the tested bile salts are representative of the multitude of bile salts produced by fish, but are not all-inclusive. Nine of sixty-eight FB neurons did not respond with neither excitation nor inhibition to bile salt odorant stimuli. The activity of each of these neurons was recorded simultaneously with the activity of at least one FB neuron that responded excitedly to bile salt odorant stimuli. Based on the location of these recorded neurons within the medial regions of the catfish FB, their EMRRs could possibly include bile salts not tested by the current investigation or other socially relevant olfactory cues. Further, the EMRRs of these neurons did not include any of the other types of stimuli (amino acids or nucleotides) tested in this investigation.

COMPARISON OF FB AND OB NEURON THRESHOLDS TO BILE SALTS

Thresholds for the detection of bile salts by FB neurons ranged from 0.01µM-0.1µM, which is ~1-2 log units lower than that reported for OB neurons in the same species (chapter 2;
Two possibilities may explain the enhancement in threshold detection in the FB: 1) convergence of neuronal input from the OB resulted in amplification of olfactory signals in FB neurons, and 2) the lower spontaneous frequency of FB neurons resulted in less “background noise” when analyzing spike trains for odorant-driven significance changes in spike frequency. Fifty-three percent of Group T FB neurons responded excitedly to bile salts at a concentration 1 log unit lower than that observed for OB neurons. Seventy-seven percent of Group N FB neurons responded to bile salts 1-2 log units lower than did OB neurons. Group G FB and OB neurons both responded to bile salt stimuli at $10^{-8}$ M; however, 60% of the recorded Group G FB neurons responded excitedly at this concentration where as only 6% of Group G OB neurons responded excitedly. FB neurons of channel catfish appear to be more sensitive to bile salt odorants than OB neurons in this species. Thresholds for the detection of bile salts reported for FB neurons are likely a more accurate estimation of the behavioral thresholds in channel catfish.

COMPARISON OF THE EMRRs OF FB AND OB NEURONS

The present investigation determined the EMRRs of 45 FB neurons in the channel catfish. Based on the EMRRs, the FB neurons were divided into three categories: 1) Group T, neurons excited by only TBS, 2) Group N, neurons excited by only NBS, and 3) Group G, neurons excited by TBS, NBS and GBS. The arrangement of molecular features present on bile salt molecules is key to determining the response of individual FB neurons. Moreover, for a particular bile salt molecule to elicit an excitatory response from a given FB neuron, the molecular features of that molecule must be included in the EMRR of that neuron. The EMRRs of the FB neurons reported here are indistinguishable from the EMRRs of OB neurons reported previously (chapter 2; Rolen and Caprio 2007). Chapter 2 presented a more detailed comparison of the EMRRs of bile salt-responsive OB neurons with both previous studies in fish and studies
involving the human plasma membrane bile salt receptor which investigated the importance of specific molecular features present on bile salt molecules; however, important points will be repeated below. Experiments in zebrafish, sea lamprey and lake char suggest that ORNs/mORs in these fish can discriminate bile salts by the molecular moieties present on the side chain and steroid nucleus (Zhang and Hara 1994; Li et al. 1995; Michel and Derbidge 1997; Li and Sorensen 1997; Friedrich and Korsching 1998; Siefkas and Li 2004). All three of these species were reported to discriminate GBS, TBS and NBS while sea lamprey and salmonids also discriminate sulfonated bile salts, as do catfish. However, there are no published reports describing the EMRRs of bile salt-responsive olfactory neurons in the CNS to which a comparison of these data can be made.

The human plasma membrane bile salt receptor discriminates bile salts by specific molecular features located along the steroid nucleus of the bile salt molecule (Maruyama et al. 2002; Kawamata et al. 2003). This receptor was reported to activate increased cAMP production when stimulated with bile salts lacking hydroxyl moieties at C7 and C12. The conjugating group at C24 was determined to be of minor importance for cAMP activation by this receptor. The molecular features at these locations were also important in determining the EMRRs for both FB and OB neurons in channel catfish. Some Group N OB (Neurons 1-8, Fig. 2.7) and FB (Neurons 1-5, Fig. 3.7) neurons showed a preference for bile salts lacking –OH moieties at C7 and C12. Also, the conjugating group at C24 proved to be less important for Group G neurons than the molecular features present at C3 and C7.

BEHAVIORAL SIGNIFICANCE OF THE OLFACTORY DETECTION OF BILE SALTS

Unfortunately, the behavioral significance for the olfactory detection of bile salts by channel catfish is currently unknown. Investigations involving salmonids and sea lamprey, however, currently provide the best evidence as to the role of bile salts in fish olfaction. Several
reports suggest that specific bile salts emanating from sea lamprey (*Petromyzon marinus*) mediate migration in this species. It is hypothesized that recognition and discrimination of specific sea lamprey bile salts from those of other species is key for a successful migration of sea lamprey to suitable spawning habitats (Li et al. 1995; Li and Sorensen 1997; Bjerselius et al. 2000; Polkinghorne et al. 2001; Vrieze and Sorensen 2001; Li et al. 2002; Fine et al. 2004; Fine and Sorensen 2005; Sorensen et al. 2005). Anadromous fishes utilize olfactory cues to locate suitable spawning areas within streams and tributaries with successive generations returning to the area where they were spawned (Hasler and Scholz 1983; Smith 1985; Stabell 1992).

Nordeng (1971, 1977) proposed that homeward migration is an inherited response to this population-specific odor learned prior to downstream migration and termed this “the pheromone hypothesis”. Both juvenile salmonids that begin migration to the sea and those which remain within the specific stream where they hatched provide a continual source of population-specific odor in the water system to which adults orient and respond. Based on electrophysiological data, bile salts were later suggested to be a component of this population-specific odor (Døving et al. 1980).
CHAPTER 4

BILE SALTS ARE EFFECTIVE TASTE STIMULI IN CHANNEL CATFISH*
INTRODUCTION

Chemosensory systems of fishes detect and discriminate biologically relevant environmental cues conveying information pertaining to conspecifics, spawning habitats and food sources (Sorensen and Caprio 1998). These chemosensory systems are uniquely different from those of terrestrial vertebrates in that both olfactory and gustatory stimuli are dissolved in aqueous solution. Specifically for teleosts, a variety of water-soluble molecules (amino acids, bile salts, nucleotides, polyamines and sex pheromones) was previously identified as potent chemosensory stimuli (Michel et al. 2003; Rolen et al. 2003; Sorensen and Caprio 1998; Caprio and Derby 2008). To better understand how chemosensory information for a given class of stimuli is processed by teleosts, investigations of both the gustatory and olfactory systems are required.

The majority of chemosensory investigations save one (Yamashita et al. 2006), which investigated the taste system of the rainbow trout, studied the detection of amino acids by the olfactory system. The current chapter presents data indicating bile salts are effective facial taste stimuli in the channel catfish. The results indicate that electrophysiological thresholds are in the low nanomolar range, and that bile salts are processed by facial neural pathways both independent from those processing amino acids and by those conveying both types of taste information.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Channel catfish (*Ictalurus punctatus*, Rafinesque), 15-22cm, were obtained from indoor recirculating tanks at the Louisiana State University (LSU) Aquaculture Facility. Fish were held in the LSU Animal Care Facility in a 300L aquarium filled with charcoal filtered tap water
(CFTW) and maintained on a 12h:12h light:dark regime for up to two weeks. The temperature of the aquarium water was held at 31°C.

ANIMAL PREPARATION

The procedures outlined are in accordance with a protocol approved by the Institutional Animal Care and Use Committee (LSU School of Veterinary Medicine).

Each catfish was immobilized with an initial intramuscular injection of Flaxedil (gallamine triethiodide, 0.03mg/100g body weight). Subsequent injections of Flaxedil were provided as needed during experimentation via a hypodermic needle embedded in the flank musculature. The catfish was wrapped in wet tissue paper and secured to a wax block in a customized Plexiglas® chamber. The gills were irrigated via a constant flow of CFTW containing the general anesthetic, MS-222 (ethyl-m-aminobenzoate methane sulfonic acid; initial concentration, 50mg/L; Sigma Chemical, St. Louis, MO) for the duration of the experiment. The local anesthetic tetracaine (3% wt/vol) was applied locally to the skin before surgical procedures to expose by deocculation a portion of the mandibular branch (ramus mandibularis trigemini; Herrick 1901; Finger 1976) of the facial-trigeminal nerve complex that innervates taste buds on the caudal portion of the maxillary barbel. A mandibular branch of the facial/trigeminal complex that innervates the caudal maxillary barbel was selected for recording since it is separate from the other large nerve branches of the complex that innervate the rostral portion of the head. Procedures for surgical exposure were previously described (Caprio 1995). Following the surgical procedures, the connective tissue encasing the nerve branch was removed and the nerve was cut at its most visible caudal point in the orbit. Depending upon the preparation, recordings were obtained from either the whole nerve, nerve twigs or from few fibers. Neural activity was recorded with a tungsten hook electrode (Fig. 4.1), ac amplified (Grass Instruments P511, Quincy, MA, USA; bandpass 30-3,000Hz), integrated (only for whole nerve and nerve twig
preparations), monitored aurally, displayed on an oscilloscope and a DC chart recorder, and stored on the audio channel of a hifi VCR.

CHEMICAL STIMULI

The test stimuli included L-amino acids (alanine [Ala], arginine [Arg] and proline [Pro]) and bile salts (sodium salts of chenodeoxycholic acid [CDC], glycochenodeoxycholic acid [GCDC], taurochenodeoxycholic acid [TCDC], and taurocholic acid [TCA]). Alanine, arginine and proline were previously shown to be highly potent taste stimuli for the channel catfish (Caprio 1978; Kohbara et al. 1992). The four bile salts tested in this study were selected to include two produced by the channel catfish (TCDC and TCA; Kellogg 1975) and two bile salts (GCDC and CDC) which have a close structural resemblance to TCDC and TCA (Fig. 4.2).

Previous investigations (Døving et al. 1980; Goh and Tamura 1980; Jones and Hara 1985; Hellstrøm and Døving 1986; Friedrich and Korsching 1998; Nikonov and Caprio 2001; Zhang et al. 2001; Rolen et al. 2003; Rolen and Caprio 2007) commonly utilized one or more of these bile salts in their studies. The molecular features of TCDC, GCDC and CDC differ only by the molecular moiety conjugated to carbon 24 (C24). TCA contains an hydroxyl group at C12 whereas this molecular feature is a –H in the other three tested bile salts. The variation in the conjugating group at C24 among these bile salts affords the ability to test three different classes of bile salts (taurine-, glycine- and non-conjugated). All four bile salts tested were 3α, 5β, 7α, and 12α isomers. All chemical stimuli were purchased from Sigma (St. Louis, MO) and were of the highest purity available (97%-99%). Stock solutions of amino acids and bile salts were prepared weekly using CFTW and were refrigerated when not in use. Test solutions were diluted daily from stock solutions to experimental concentrations with CFTW and were tested at room temperature, the same as that of the water flow to the maxillary barbel.
Figure 4.1. A schematic of the experimental setup for facial nerve recordings. The position of the hook electrode is indicated with a red circle. Stimuli were applied to the maxillary barbel via polyethylene tubing into a glass sleeve housing the maxillary barbel. Modified from (Atema 1971).
Figure 4.2. The molecular formulae of the bile salts tested. Molecular features, designated by R1 and R2, of each bile salt tested vary at carbon positions C12 of the steroid backbone and C24 of the side-chain, respectively. The stimuli included different classes of bile salts based on the specific molecular feature (R2) attached to C24 (glycine-conjugated [GBS], taurine-conjugated [TBS], non-conjugated [NBS]). All of the bile salts above are 3α, 5β, 7α, and 12α isomers. An asterisk indicates those bile bile salts produced by the channel catfish (Kellogg 1975).
STIMULUS DELIVERY

Stimulus delivery was via a “gravity-feed” system that was previously described (Sveinson and Hara 2000). The maxillary barbel was inserted into a glass sleeve and continuously bathed in CFTW (flow rate, 8-10ml/min) not containing MS-222, or during cross-adaptation experiments (see below), continuously bathed by the adapting solution. Briefly, stimulus solutions and the CFTW used to bathe the maxillary barbel were delivered through separate Teflon® tubes (diameter 0.8mm) to a common tube that extended 46 cm to the maxillary barbel. A foot switch connected to an electronic timer (Model 645, GraLab Instruments Division, Dimco-Gray Corporation, Centerville, OH) triggered a pneumatic actuator valve to introduce the stimulus for 2 s applications. With the sole exception of when a stimulus was added, CFTW alone continuously perfused the maxillary barbel to 1) prevent desiccation, 2) facilitate stimulus delivery, 3) avoid the introduction of mechanical artifacts associated with stimulus presentation and 4) rinse the glass sleeve containing the maxillary barbel clear of any residual stimuli for a minimum of 2 mins between stimulus applications.

CROSS-ADAPTATION EXPERIMENTS

Electrophysiological cross-adaptation experiments to determine the relative independence of the neural pathways for the stimuli consisted of three stages: (A) Pre-adaptation: CFTW continuously bathed the left maxillary barbel for a minimum of 5 mins prior to stimulus applications. Bile salts and amino acids were tested at $10^{-5}$M and $10^{-6}$M, respectively. CFTW served as the control during pre-adaptation. (B) Adaptation: the adapting solution continuously bathed the maxillary barbel. All stimuli tested during adaptation were dissolved in the adapting solution. The adapting solution served as the control and was tested immediately prior to each test stimulus. If responses to the test stimuli were suppressed to the control level (complete adaptation), these test stimuli were considered to share the same neural pathways as the adapting
stimulus. If the responses to test stimuli were significantly greater than the control level, these test stimuli were considered to have at least partially independent receptor sites and neural pathways from the adapting stimulus. (C) Post-adaptation, CFTW continuously bathed the maxillary barbel for 5 mins prior to stimulus application. Stimuli and controls were identical to those described during pre-adaptation.

RESULTS

CHARACTERISTICS OF THE INTEGRATED TASTE RESPONSES TO BILE SALTS

Initially, integrated multiunit responses to bile salts were recorded from the entire branch of the facial/trigeminal nerve complex innervating the caudal portion of the maxillary barbel; however, it is the facial nerve components from which taste activity is recorded. These recordings permitted an evaluation of the stimulatory effectiveness of bile salts relative to that for amino acids, the more well-established tastants for channel catfish (Caprio 1978; Kohbara et al. 1992). Only prominent phasic responses were evident to both classes of stimuli (Fig. 4.3); the neural activity increased as the stimuli contacted the maxillary barbel and returned to pre-stimulus levels without any obvious tonic level of activity. A stimulus duration of 2 s was chosen for the remaining experiments. Dose-response data indicate that thresholds for the more effective bile salts tested were as low as $10^{-11}$-$10^{-10}$M, with the magnitude of the integrated response generally increasing with stimulus concentration up to approximately micromolar concentration (Figs. 4.4, 4.5). The four tested bile salts ($10^{-6}$M TCDC, TCA, CDC and GCDC) evoked mean responses ≤50% of that evoked by the standard, $10^{-6}$M L-alanine (Fig. 4.5).

CROSS-ADAPTATION EXPERIMENTS: EVIDENCE FOR THE RELATIVE INDEPENDENCE OF RECEPTOR SITES FOR AMINO ACIDS AND BILE SALTS

During continuous application of $10^{-5}$M TCDC to the maxillary barbel, the integrated responses to $10^{-5}$M GCDC and CDC were reduced to 12.8±11.1% and 11.1±10%, respectively
(i.e. control level; one-way ANOVA; Tukey’s post hoc test, P>0.05) of their unadapted responses while responses to L-amino acids were unaffected (Figs. 4.6A, 4.7A). During continuous application of $10^{-6}$M L-amino acids (alanine, arginine and proline), the integrated multiunit responses to $10^{-5}$M bile salts were reduced to only ~69.7%-81.9% of their unadapted responses (Figs. 4.6B, 4.7B).

NERVE TWIG AND UNIT DATA: EVIDENCE FOR BOTH DISTINCT AND SHARED NEURAL PATHWAYS IN THE FACIAL NERVE

To test for the possibility of independent neural pathways for bile salts and amino acids, the nerve innervating the caudal portion of the maxillary barbel was carefully teased into multiple bundles. We reasoned that if separate neural pathways for the transmission of bile salt and amino acid information existed within this nerve and the relative number of fibers most responsive to these stimuli differed across the bundles, then the ratio of taste responses to bile salts and amino acids would also differ across the tested nerve bundles. A total of eighteen nerve bundles from three channel catfish were tested with a mixture of $10^{-5}$M bile salts (TCDC, TCA, GCDC and CDC) and a mixture of $10^{-6}$M L-amino acids (Ala, Arg and Pro). As hypothesized, the integrated response to bile salts varied in comparison to that for amino acids across the 18 nerve twigs tested (Fig. 4.8). The integrated taste responses elicited by the bile salt mixture were averaged and expressed as a percentage of the averaged integrated taste response to the amino acid mixture obtained from each nerve twig. The magnitude of the integrated response recorded to the bile salt mixture ranged from 0% to greater than 100% of the response to the mixture of $10^{-6}$M amino acids (Table 4.1). The response to amino acids and not to bile salts for at least a portion of the twig data (Fig. 4.8A) confirms the independence of both receptor sites and at least a portion of the neural pathways for the tested stimuli.
Figure 4.3. Integrated whole nerve taste responses to (A) $10^{-6}$M L-alanine and (B) $10^{-5}$M TCDC. Each compound was tested at two stimulus durations, 2 s and 5 mins. Note that each compound evoked only phasic responses regardless of stimulus duration.
Figure 4.4. Integrated taste responses to $10^{-11} - 10^{-4}$M TCDC recorded from the entire branch of the facial/trigeminal complex that innervates the caudal portion of the maxillary barbel. Responses to CFTW (C) control and $10^{-6}$M L-Ala are shown to allow comparisons.
Figure 4.5. Dose-response plots of integrated taste responses to bile salts standardized to the response to $10^{-6}$M L-alanine. The number of fish tested (N) is provided in the figure legend. The averaged control magnitude value was subtracted from the averaged stimulus magnitude response at each concentration. Data points and error bars, mean ± s. e. m.
**Figure 4.6.** Representative cross-adaptation experiments illustrating the integrated taste activity recorded (1) prior to, (2) during and (3) after adaptation to (A) $10^{-5}$M TCDC and (B) a mixture of $10^{-6}$M L-amino acids (Ala, Arg and Pro). The adapting solution is underlined.
Figure 4.7. Results of cross-adaptation experiments. (A) Adaptation to $10^{-5}$M TCDC; (B) Adaptation to a mixture of $10^{-6}$M amino acids (alanine, arginine and proline). Bars indicate the percentage of the unadapted response (mean ± s. d.). Responses significantly greater than control responses: (A) Pro, Ala and Arg, (B) TCDC, GCDC and CDC (one-way ANOVA; Tukey’s post hoc test, P<0.05). N=3 fish tested.
Figure 4.8. Integrated multiunit taste recordings from three separate facial nerve twigs (A-C) innervating the maxillary barbel in a single fish showing the variability of the magnitude of the integrated responses to bile salts with respect to amino acids. A) Nerve twig lacking a bile salt response but showing a large magnitude amino acid response. B) Nerve twig with a significant bile salt response and a greater magnitude amino acid response. C) Nerve twig responding approximately equally to the bile salt and amino acid mixtures. C= CFTW control, BS= a mixture of $10^{-5}$M TCDC, TCA, GCDC and CDC, AA= a mixture of $10^{-6}$M L-Ala, L-Arg and L-Pro. Scale bar provided in A.
A few (n=11) single fibers were also isolated and tested with bile salts and amino acids to further investigate the specificities of the neural pathways for these stimuli. From a total of eleven single fibers obtained, two were excited solely by bile salts (Fig. 4.9A), five solely to amino acids (Fig. 4.9B) and four fibers were excited by both types of stimuli (Fig. 4.9C).

Table 4.1. The number of nerve twigs recorded whose response magnitude to bile salts is expressed as a % of the response magnitude to the mixture of 10^6M amino acids.

<table>
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<th>Response magnitude</th>
<th>0%-%20%</th>
<th>20%-%40%</th>
<th>40%-%60%</th>
<th>60%-%80%</th>
<th>80%-100%</th>
<th>&gt;100%</th>
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<tr>
<td># of nerve twigs</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
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DISCUSSION

RESPONSE CHARACTERISTICS TO BILE SALTS

Electrophysiological responses to bile salts were recorded from the facial nerve fibers that innervate taste buds and reside within the branch of the facial/trigeminal complex that innervates the caudal maxillary barbel. Integrated multiunit activity in response to bile salts was fast-adapting, displaying only a phasic response irrespective of stimulus duration which is similar to that obtained for amino acids in both this report and previously (Caprio 1978).

However, a phasic only response to bile salts in the channel catfish is in direct contrast to responses to bile salts in rainbow trout where both phasic and tonic components of the gustatory response were clearly evident (Yamashita et al. 2006).

DOSE-RESPONSE PROPERTIES

Dose-response plots of the integrated multiunit taste activity demonstrate that taste thresholds to three of the four bile salts tested were ~10^{-11}M-10^{-10}M. Of the two bile salts tested
**Figure 4.9.** Responses of single facial taste fibers. (A) A single fiber responding only to the bile salt (BS) mixture (10^{-5} M TCDC, TCA, GCDC and CDC); (B) a single fiber responding only to the amino acid (AA) mixture (10^{-6} M Ala, Arg and Pro); (C) a single fiber responding to both stimulus mixtures. A and B were recorded simultaneously from different fibers in the same preparation while Unit #3 was recorded from a different animal. Horizontal bars indicate 2s stimulus applications. Clusters of individual spike trains are shown in insert I and II to indicate that the action potentials recorded in A-C were evoked by single fibers. Ct = CFTW.
TCDC and TCA) that are produced by channel catfish, TCDC was one of the more effective taste stimuli. TCA was a relatively poor stimulus compared to both TCDC and to the two additional bile salts tested, CDC and GCDC, that are not produced by channel catfish. The data indicate that thresholds to bile salts are lower than those for amino acids in the facial taste system in the same species (Kohbara et al. 1992) and 1-2 log units higher than that recorded to TCA in the rainbow trout (Yamashita et al. 2006). Although thresholds to bile salts were lower than to amino acids in the facial taste system, amino acids elicited greater integrated response magnitudes at equivalent stimulus concentrations. The magnitude of the response to the standard, $10^{-6}$M L-Ala, was typically twice that to the more potent bile salts tested. In all recordings from the entire nerve branch innervating the caudal maxillary barbel, the responses to $10^{-6}$M bile salts never exceeded ~50% of the response to $10^{-6}$M L-Ala.

Currently, there are no published reports of comparable data investigating the stimulatory effectiveness of bile salts to olfactory receptor neurons in channel catfish. However, single olfactory bulb neurons in this species responded excitedly to bile salts between $10^{-7}$M and $10^{-6}$M (Rolen and Caprio 2007). Thus, the gustatory system of channel catfish is ~1,000-10,000 times more sensitive to this class of molecules than its olfactory counter-part. In comparison, olfactory and gustatory thresholds to bile acids in salmonids may not be so disparate as olfactory thresholds for the more stimulatory bile acids in salmonids estimated from integrated olfactory bulb waves ranged between $10^{-9}$M and $10^{-11}$M (Døving et al. 1980), whereas taste threshold to taurocholic acid in rainbow trout was $10^{-12}$M (Yamashita et al. 2006).

**TASTE RECEPTOR SITES FOR BILE SALTS**

Cross-adaptation experiments in the present study indicated the relative independence of taste receptor sites for bile salts and amino acids which is similar to that reported for rainbow trout (Yamashita et al. 2006). The cross-adaptation data also suggest that the three bile salts...
tested individually bind to the same receptor since adaptation with TCDC eliminated to control level the responses to CDC and GCDC. These results are also similar to that observed in the rainbow trout (Yamashita et al. 2006). However, since single olfactory bulb neurons in the channel catfish could discriminate among different molecular features of specific bile salts (Rolen and Caprio 2007), it is possible that relatively independent taste receptor sites exist for other untested biliary steroids.

BILE SALT AND AMINO ACID TASTE INFORMATION IS PROCESSED BY BOTH INDEPENDENT AND SHARED NEURAL PATHWAYS

The present nerve twig and single fiber data suggest that both independent and shared neural taste pathways exist for bile salts in the channel catfish. Small teased branches of the nerve innervating the caudal maxillary barbel were responsive to amino acids and not bile salts. Further, single fiber data confirmed that a portion of the facial nerve neural pathways conveying bile salt taste information is separate from those pathways conveying amino acid taste information as evidenced by single fibers responsive only to the bile salt or to the amino acid mixtures. However, single fibers responding excitedly to both the bile salt and amino acid mixtures were also observed suggesting some degree of overlap also occurs. Single taste fibers responding to structurally different classes of tastants were previously demonstrated for Seriola quinqueradiata where single palatine taste fibers responded to both amino acid and nucleotide stimuli (Zeng and Hidaka 1990).

BEHAVIORAL IMPLICATIONS

The present study combined with data from a previous investigation (Yamashita et al. 2006) indicates that channel catfish and rainbow trout possess gustatory systems capable of detecting bile salts. Currently, there are no published investigations citing specific behaviors resulting from gustatory detection of bile salts in either fish. To date, the olfactory detection of
bile salts and its role in sea lamprey migration is the most well-documented case of a direct effect of biliary steroids on the behavior of a fish. It is hypothesized that olfactory recognition and discrimination of specific sea lamprey bile salts are key for successful migration of adult sea lamprey to suitable spawning habitats. Sexually mature sea lampreys innately recognize a mixture of species-specific bile salts (Li et al. 1995; Li et al. 2002; Fine et al. 2004; Sorensen et al. 2005) and select for streams containing populations of sea lamprey larvae, which would indicate suitable spawning grounds (Bjerselius et al. 2000; Polkinghorne et al. 2001; Vrieze and Sorensen 2001; Fine and Sorensen 2005). Previous investigations demonstrated that freshwater eels (Sola and Tosi 1993), Artic char (Jones and Hara 1985) and cod (Hellstrøm and Døving 1986) respond to synthetic bile salts with activities classified as orientation and snapping. Further, Hellstrøm and Døving (1986) showed that TCA was detected in the absence of a functioning olfactory system. Future behavioral investigations are needed to determine the role of gustation in the detection of bile salts for both channel catfish and other species.
CHAPTER 5

SUMMARY AND CONCLUSIONS
The aqueous environment in which teleosts live provides an ever-present milieu of chemosensory stimuli having similar solubility properties. Unlike terrestrial vertebrates whose olfactory system is designed to detect air-borne odors and whose gustatory system detects water-soluble compounds, both odorants and tastants for teleosts are water-soluble molecules. Evolution has dictated that both systems sample the same medium for relevant chemosensory cues. Therefore, one environment provides a single medium in which both odorants and tastants are present in abundance. Previous electrophysiological investigations established amino acids as being both olfactory and gustatory stimuli (reviewed in Hara 1994; Sorensen and Caprio 1998); yet behavioral studies show that each system drives unique behavioral responses (Atema 1971; Valentincic et al. 1994). The key to understanding how a chemosensory system processes stimuli into a meaningful behavioral output is to learn how single neurons within the system respond to individual relevant stimuli.

The goal of chapters two and three was to determine the EMRRs of single OB and FB neurons, respectively. The EMRR is the range of molecules that elicits excitatory responses from a given neuron which would drive excitatory responses in postsynaptic targets. The results of this investigation indicate that at least four molecular features present on the steroid backbone and side chain of bile salt molecules lie within the EMRRs of OB and FB and are critical in determining the neuron’s response to each molecule. EMRRs of both OB and FB neurons of channel catfish to bile salts were shown to be highly similar although the thresholds of detection of bile salts for FB neurons were 1-2 log units lower than OB neurons. In chapters two and three, comparisons were made between the recorded EMRRs and previous studies in fishes investigating the odorant selectivities of ORNs. These combined investigations suggest fish olfactory systems detect taurine-, glycine- and non-conjugated bile salt information through separate populations of ORNs and the integrity of this information is maintained by OB and FB
neurons. Surprising similarities were also observed between the recorded EMRRs of OB and FB neurons and the human plasma-membrane bile salt receptor (TGR5/BG37). TGR5/BG37 preferentially responded to bile salts lacking –OH moieties at C7 and C12 and the conjugating group at C24 appears to have a minor influence on cAMP production. The molecular features present at C7 and C12 were important in determining the EMRRs of Group G OB and FB neurons while the conjugating moiety at C24 also played a minor role.

In chapter four, data were presented indicating that bile salts are gustatory stimuli to channel catfish. The data indicate that independent taste pathways for bile salts exist alongside those transmitting both bile salt and amino acid taste information. The facial taste system of the channel catfish, however, may not discriminate individual bile molecules as cross-adaptation experiments indicate that a common receptor for bile salts exists. It thus appears that the sensory discrimination of bile salts is accomplished primarily by the olfactory system in this species. Surprisingly, however, the facial taste system of the channel catfish is more sensitive to bile salts than even the olfactory system as detection thresholds for bile salts by the facial taste system are 2-3 log units lower than those for olfactory FB neurons.

It is clear from the data reported in the present study that channel catfish detect bile salts through both their olfactory and gustatory systems, but discriminate individual bile salt molecules by their olfactory system. Although the behavioral significance for the detection of bile salts is currently unknown for channel catfish, these molecules were shown to be behaviorally relevant stimuli mediating orientation, migration and homing behaviors in other fish species (Bjerselius et al. 2000; Polkinghorne et al. 2001; Fine and Sorensen 2005; Hellstrom and Døving 1986; Jones and Hara 1985; Sola and Tosi 1993; Vrieze and Sorensen 2001). The behaviors listed above are generally considered social behaviors in fish and are thought to be mediated exclusively by the olfactory system. The question then arises why are social stimuli
detected by the taste system? Taste systems of fish were previously shown to mediate ingestive behaviors such as snapping and swallowing (Lamb and Finger 1995; Valentincic et al. 1994). Channel catfish are a piscivorous species feeding primarily on smaller fish. Bile salts emanating from these prey fish could constitute a portion of the chemical signal which the channel catfish would utilize to identify, search out and ultimately consume smaller fish species.

The present research opens a number of avenues for future researchers to build upon. Presently, the behavioral relevance of bile salts for channel catfish is unknown which precludes any attempts to relate directly the results of the present manuscript to specific behaviors in this species. Future behavioral studies are needed to determine the behaviors elicited by bile salts through the olfactory and the gustatory systems, respectively. It is possible that the olfactory and the gustatory systems could independently mediate distinct behaviors depending on the context in which these stimuli are detected. How will the catfish respond behaviorally when the olfactory system denotes bile salts as social cues and the taste system interprets bile salts as feeding stimuli? Further, the results of this dissertation suggest that although single olfactory neurons discriminate individual bile salt molecules, the facial taste system cannot. Can the channel catfish behaviorally discriminate individual bile salt molecules?

Since both OB and FB neurons respond similarly to bile salts, another important avenue for future research would be to determine the response specificity to these compounds of even higher order neurons within the olfactory pathway. Currently, little is known about the downstream targets of the olfactory FB neurons described in Chapter three. A combination of anatomical tracing experiments describing the location of these postsynaptic targets and electrophysiological experiments detailing the EMRRs of these neurons would further our understanding of the olfactory processing of bile salt stimuli. Finally, how central gustatory neurons respond to bile salts is another avenue of research awaiting discovery.
REFERENCES


APPENDIX: LETTERS OF AUTHORIZATION TO REPRINT
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Subject: Re: JEB article

Dear Shane,

Permission is granted - and good luck with the thesis.

Best wishes, Claire Moulton.

This email was sent by Claire Moulton on behalf of the Sales & Marketing team.

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Date: Tue, 7 Oct 2008 10:46:35 -0500
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To whom it may concern,
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Sincerely,
Shane Rolen
VITA

Shane Howell Rolen was born to Eugene and Jeanette Rolen in 1978, in Monroe, Louisiana. He obtained a Bachelor of Science degree with a minor in chemistry in May of 2000 from the University of Louisiana at Monroe. Shane enrolled at Louisiana State University in August of 2000 pursuing a Master of Science degree in Dr. John Caprio’s laboratory where he studied polyamines as olfactory stimuli in goldfish, Carassius auratus. After completing his master’s, Shane continued research as a doctoral candidate in Dr. John Caprio’s laboratory focusing on characterizing bile salts as both odorants and tastants for the channel catfish, Ictalurus punctatus. Shane will complete his studies and earn the degree Doctor of Philosophy in December of 2008.