*J Neurophysiol* 97: 4058–4068, 2007.
First published April 18, 2007; doi:10.1152/jn.00247.2007.

# Processing of Bile Salt Odor Information by Single Olfactory Bulb Neurons in the Channel Catfish

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Submitted 6 March 2007; accepted in final form 13 April 2007

**Rolen SH, Caprio J.** Processing of bile salt odor information by single olfactory bulb neurons in the channel catfish. *J Neurophysiol* 97: 4058–4068, 2007. First published April 18, 2007; doi:10.1152/jn.00247.2007. A chemotopic map of biologically relevant odorants (that include amino acids, bile salts, and nucleotides) exists in the olfactory bulb (OB) of channel catfish, *Ictalurus punctatus*. Neurons processing bile salt odorant information lie medially within this OB map; however, information as to how single neurons process bile salt odorant information is lacking. In the present report, recordings were obtained from 51 OB neurons from 30 channel catfish to determine the excitatory molecular receptive range (EMRR) of bile salt responsive neurons. All recordings were performed in vivo within the medial portions of the OB using extracellular electrophysiological techniques. Excitatory thresholds to bile salts typically ranged between 0.1 and 10 $\mu$M. The bile salt specificity of OB neurons were divided into three groups: neurons excited by taurine-conjugated bile salts only (group T), neurons excited by nonconjugated bile salts only (group N), and neurons excited by at least one member of each of the three classes of bile salts tested (group G). In addition to the conjugating group at C24 of the side-chain, OB neurons discriminated bile salts by the molecular features present at three other carbon positions (C3, C7, and C12) along the steroid backbone. These data suggest that OB neurons are selectively excited by combinations of molecular features found on the side-chain and along the steroid nucleus of bile salt molecules.

## INTRODUCTION

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. Behavioral studies indicate that the detection and discrimination of bile salts by the olfactory system mediates homeward migration in anadromous fishes and sea lamprey (Jones and Hara 1985; Li et al. 2002; Sorensen et al. 2005). Bile salts also elicit snapping and orientation responses in teleosts (Hellstrom and Døving 1986; Sola and Tosi 1993).

Olfactory systems confer the ability to detect and discriminate a vast number of biologically relevant compounds that aid in identifying and locating food sources, conspecifics, mates, and spawning habitats. Initially, the olfactory system discriminates odorants with an assortment of molecular olfactory receptors (ORs) located within the ciliary and microvillar membranes of olfactory receptor neurons (ORNs). Vertebrate molecular ORs are members of the superfamily of 7-transmembrane domain (7-TMD) G-protein-coupled receptors (Buck and Axel 1991). Although coexpression 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 1 of ~1,000 molecular OR genes in mammals (Buck and Axel 1991) or one of ~100 in fish (Barth et al. 1996; Ngai et al. 1993), which encode for receptor protein molecules. ORNs expressing a particular molecular OR 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 OB, termed glomeruli, where they make synaptic contacts with dendrites of mitral/tufted neurons.

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 an 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), carp (Hamdani and Døving 2003; Hamdani et al. 2000, 2001; 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. The lateral OB processes feeding cues, such as amino acids and nucleotides, whereas social cues, such as bile salts and putative pheromones, are processed by the medial OB. In addition, 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). Unlike mammals, which have both main olfactory structures and accessory olfactory structures, the olfactory system of fishes does not

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contain the latter; therefore both feeding and social cues are processed simultaneously within the main olfactory system.

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 molecular ORs that activate the $G\alpha_{olf}$/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 (Hamdani and Døving 2003; Hamdani et al. 2000, 2001; Kyle et al. 1987; Lastein et al. 2006; Sorensen et al. 1991), comprise portions of the medial olfactory tract that project to medial regions of the olfactory forebrain (Bass 1981; Finger 1975; Nikonov et al. 2005).

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.

The present study investigates 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. 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). EMRR is defined as the range of odorant molecules that elicit excitatory responses from a given neuron. We report that 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.

## METHODS

### *Experimental animals*

Channel catfish (*Ictalurus punctatus*), 15–22 cm, 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 12 h:12 h light:dark regime for ≤2 wk. For those fish gathered from floating cages, the water temperature was held <20°C during the winter months and >31°C 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 20°C.

### *Animal preparation*

The procedures outlined in the following text 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 gallamine triethiodide (Flaxedil, 0.03 mg/100 g body wt). 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, 50 mg/l; Sigma Chemical, St. Louis, MO). Tetracaine (3%) was applied to the surgical site 5 min 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.

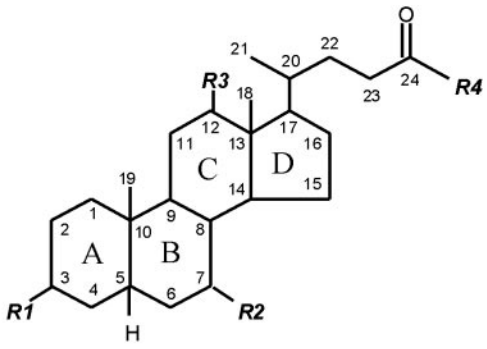
### *Stimulus solutions and delivery*

The odorants included L-amino acids (alanine, arginine, glutamate and methionine), bile salts [Fig. 1; 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 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 (−20°C) until just prior to testing. For particular bile salts either 0.4% methanol or 0.4% ethanol were initial solvents. 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: 0.8 mm) 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 2-s applications. CFTW continuously perfused the olfactory mucosa to prevent the mucosa from desiccating, facilitate stimulus delivery, avoid the introduction of mechanical artifacts associated with stimulus presentation, and rinse the olfactory organ clear of any residual stimuli for a minimum of 2 min between stimulus applications.

### *Electrophysiological recording techniques*

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-agar-filled capillary pipettes (Caprio 1995). The pipette of the active electrode was positioned near the midline raphe




| | Compound | *R1* (C3) | *R2* (C7) | *R3* (C12) | *R4* (C24) |
|---|---|---|---|---|---|
| GBS | Glycochenodeoxycholic acid (GCDC) | OH | OH | H | $NHCH_2COOH$ |
| | Glycocholic acid (GCA) | OH | OH | OH | $NHCH_2COOH$ |
| TBS | Taurochenodeoxycholic acid (TCDC) | OH | OH | H | $NHCH_2CH_2SO_3H$ |
| | Taurocholic acid (TCA) | OH | OH | OH | $NHCH_2CH_2SO_3H$ |
| | Taurodeoxycholic acid (TDC) | OH | H | OH | $NHCH_2CH_2SO_3H$ |
| | Taurolithocholic acid (TLC) | OH | H | H | $NHCH_2CH_2SO_3H$ |
| | Taurolithocholic acid 3-sulfate(TLCS) | $SO_4H$ | H | H | $NHCH_2CH_2SO_3H$ |
| NBS | Cholic acid (CA) | OH | OH | OH | OH |
| | Deoxycholic acid (DCA) | OH | H | OH | OH |
| | Lithocholic acid (LCA) | OH | H | H | OH |
| | Chenodeoxycholic acid (CDC) | OH | OH | H | OH |

FIG. 1. 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 stimuli included different classes of bile salts based on the specific molecular feature (R4) attached to C24 [glycine-conjugated (GBS), taurine-conjugated (TBS), nonconjugated (NBS)]. The order and types of bile salt stimuli indicated here are followed for Figs. 5, 7, and 9. All of the bile salts above are 3$\alpha$, 5$\beta$, 7$\alpha$, and 12$\alpha$ isomers.

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, West Warwick, RI), displayed on an oscilloscope and DC chart recorder, digitized and stored on a video channel of a hifi VCR.

SINGLE-NEURON RECORDINGS FROM THE OB. Single OB neuron activity, presumably from mitral cells (Kang and Caprio 1995a), was recorded extracellularly from the medial regions of the OB with low impedance (0.5–2 M$\Omega$) platinum and gold-plated, metal-filled, glass micropipettes (modified from Caprio 1995; Gesteland et al. 1959). Soda lime glass [1.1–1.2 mm ID, thin wall (0.2 mm)] was pulled on a vertical puller (Narishige PP-83) to provide a 2 $\mu$m 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 3–10 s (1.5-mV battery through a 10-M$\Omega$ resistor) with gold (code 3023, Sifco, Cleveland, OH) to form a 2- to 5-$\mu$m ball followed by a Pt (5% Pt chloride) coating electroplated (5–10 s through a 50-M$\Omega$ resistor) over the gold. The electrode was mounted on a hydraulic microdrive and advanced vertically downward from the dorsal surface of the OB. Recordings began once a spontaneously active neuron 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; band-pass: 30–3,000 Hz), observed with an oscilloscope and stored as an analog signal on an audio channel of a hifi VCR.

DATA ANALYSIS Responses of single OB neurons were classified as excitatory, suppressive, or null (not significantly different from prestimulus) based on the one-tailed interrupted time-series analysis (ITSA) (Crosbie 1993; Hudson 1977; Kang and Caprio 1995a,b). The ITSA compares statistically the number of action potentials occurring within successive 250-ms 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 ($P \leq 0.05$) than spike counts 2 s immediately prior to stimulus onset, were classified as excitatory responses.

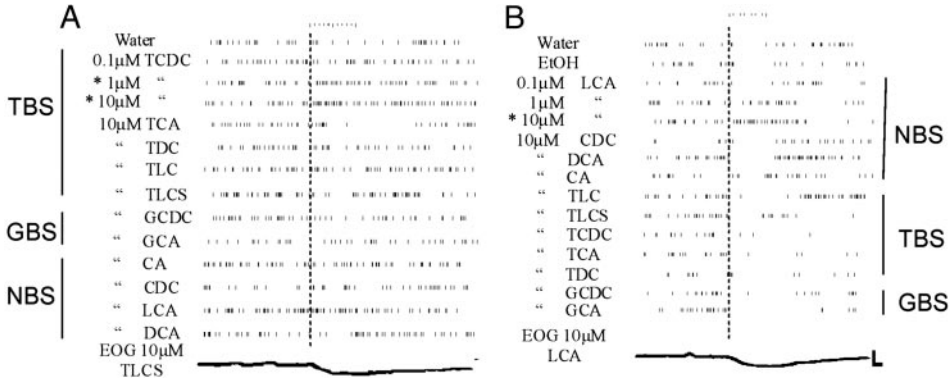



FIG. 2. Representative raster plots of group T and N olfactory bulb (OB) neurons. Two single-unit recordings are shown: a group T neuron responding excitedly to taurochenodeoxycholic acid (TCDC, a taurine-conjugated bile salt; *A*) and a group N OB neuron responding excitedly to lithocholic acid (LCA, a nonconjugated bile salt; *B*). Odorants eliciting an excitatory response ($P \leq 0.05$) are marked (*). Two second odor applications are indicated by the scale bar above each set of raster plots beginning at ···. Electroolfactogram (EOG) scale bar: 0.5 mV, 400 ms. GBS, glycine-conjugated bile salt; TBS, taurine-conjugated bile salt; NBS, nonconjugated bile salt.

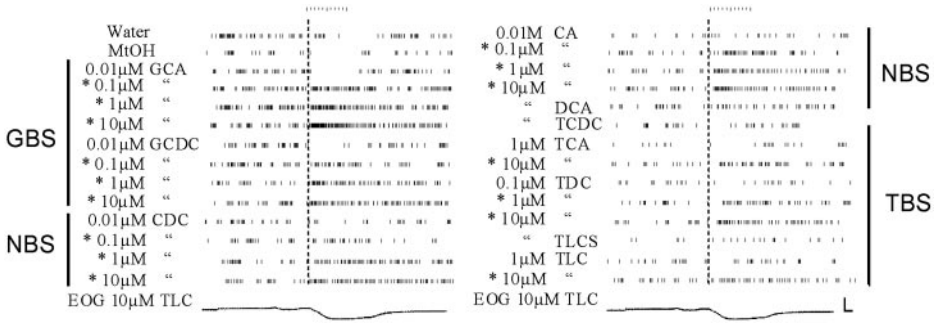



FIG. 3. A group G neuron responding excitedly to taurine-conjugated, nonconjugated, and glycine-conjugated bile salts. Odorants eliciting an excitatory response ($P \leq 0.05$) are marked (*). Two second odor applications are indicated by the scale bar above each set of raster plots beginning at ···. EOG scale bar: 0.5 mV, 400 ms.

## RESULTS

### *Molecular features of the tested bile salt odorants*

The molecular features (R1–R4) of the 11 bile salts tested differed at three carbon positions, C3, C7, and C12, along the steroid backbone and at C24 of the side-chain (Fig. 1). The 11 bile salts tested were segregated into three major categories based on the molecular feature at C24: taurine-conjugated (TBS), glycine-conjugated (GBS), or nonconjugated (NBS); 5 of the 11 bile salts were conjugated to taurine, 2 were conjugated to glycine, and 4 were nonconjugated, 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. 1). 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 3$\alpha$, 5$\beta$, 7$\alpha$, and 12$\alpha$ 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 neurons were excited by bile salt odorants $\leq$10 $\mu$M and not by 1–10 $\mu$M L-amino acids or 10–100 $\mu$M 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 (2 s) was significantly greater ($P \leq 0.05$) than that occurring spontaneously immediately prior (2 s) to stimulus application] were critically analyzed because it is the excitatory response that drives downstream neurons. However, a few findings addressing suppressive responses are reported in the following text.

In searching for bile-salt-responsive OB neurons, separate mixtures composed of TBS, NBS, and GBS, 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 all three mixtures were tested individually. Each stimulus was repeated two to seven times over the course of the experiment. Odorants eliciting excitatory responses were tested four to seven times, whereas odorants eliciting inhibitory responses were tested two to four times. In all cases studied, single neurons that responded excitedly to a particular mixture also responded excitedly to at least one component of the mixture. Further, for mixtures tested that did not elicit excitatory responses, the components of these mixtures when tested individually also did not produce an excitatory response. The 51 recorded bile salt responsive neurons were divided into three major groups based on their respective EMRRs. Eighteen of 51 (35%) neurons were classified as group T the EMRRs of which included only taurine-conjugated bile salts (Fig. 2*A*). Seventeen of 51 (33%) neurons were classified as group N the EMRRs of which included only nonconjugated bile salts (Fig. 2*B*). The remaining 16 neurons (32%) were classified as group G the EMRRs of which 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. 3).

### *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. 4), which was previously indicated to process bile salt information (Nikonov and Caprio 2001). The

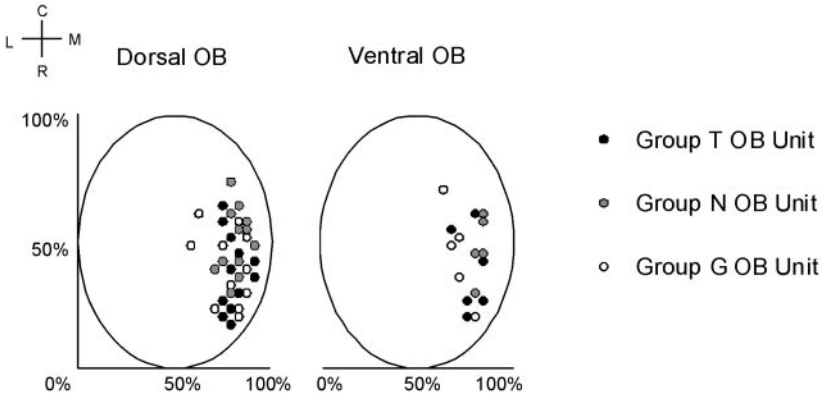



FIG. 4. Group T, N, and G neurons are distributed along both the rostral/caudal and dorsal/ventral portions of the medial region of the channel catfish OB. Stereotaxic methods were utilized to determine the location of each neuron as described previously by Nikonov and Caprio (2001). The position of each neuron depicted above is expressed as a percentage of the total length/width of the OB.

TABLE 1. *OB neuron thresholds to bile salts*

| Odorant Concentration | Number of Group T Neurons Excited by TBS ($n$ = 18) | Number of Group N Neurons Excited by NBS ($n$ = 17) | Number of Group G Neurons Excited by GBS, NBS, and TBS ($n$ = 16) | Totals of Groups T, N, & G (51 neurons) |
|---|---|---|---|---|
| $\leq 10^{-8}$ | 0(0) | 0(0) | 1(6) | 1(2) |
| $10^{-7}$ | 2(11) | 0(0) | 3(19) | 5(10) |
| $10^{-6}$ | 12(67) | 2(12) | 15(94) | 29(57) |
| $10^{-5}$ | 18(100) | 17(100) | 16(100) | 51(100) |

Parentheses enclose percentages. OB, olfactory bulb; TBS, NBS, GBS, taurine-conjugated, non conjugated, and glycine-conjugated bile salts, respectively.

data do not suggest that the bile-salt-responsive region of the channel catfish OB is subdivided into distinct subregions of neurons that selectively process each of the three types of bile salts tested.

### *Group T OB neurons*

All of the 18 group T neurons were excited by bile salts with particular combinations of molecular features where R4 was a taurine moiety (Fig. 2*A*); 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 (nonconjugated). Excitatory thresholds ranged from 0.1 to 10 $\mu$M (Table 1). Sixty-seven percent (12 of 18 neurons) of group T neurons were excited by TBS at 1 $\mu$M and 11% (2 of 18 neurons) responded at 0.1 $\mu$M.

Thirteen group T neurons were excited by only one TBS (Fig. 5); the remaining five neurons responded excitedly by 2–3 TBS. These neurons discriminated further taurine-conjugated bile salts by molecular features (R1–R3) at C3, C7, and C12 of the steroid backbone (Fig. 5). 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 (Figs. 5, neurons 1–6, and 6*A*). 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, 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 (Figs. 5, neurons 7–10, and 6*B*), 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. 5). 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 TBS.

### *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 1 to 10 $\mu$M. All group N neurons responded excitedly to at least one of the 4 tested nonconjugated bile salts at 10 $\mu$M, whereas 12% (2 of

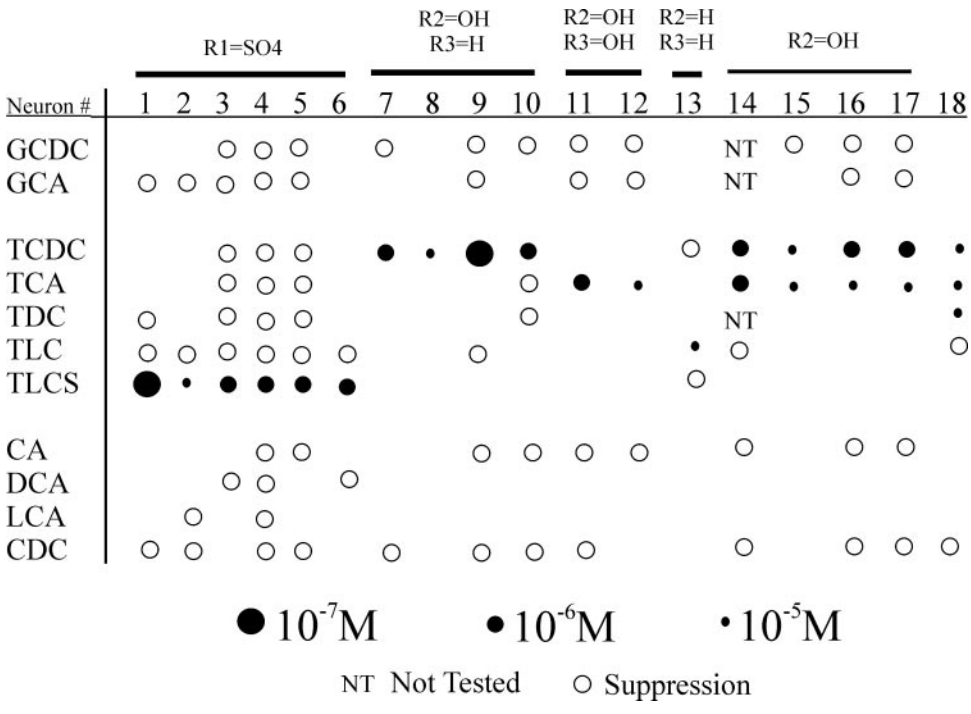



FIG. 5. Group T OB neurons are excited by only TBS. ●, excitatory responses. ○, inhibitory responses. No symbol, OB neuron activity to the odorant did not change significantly from prestimulus levels. The molecular features determined to be critical for an eliciting excitatory responses are indicated above, excluding R4, which was a taurine moiety.

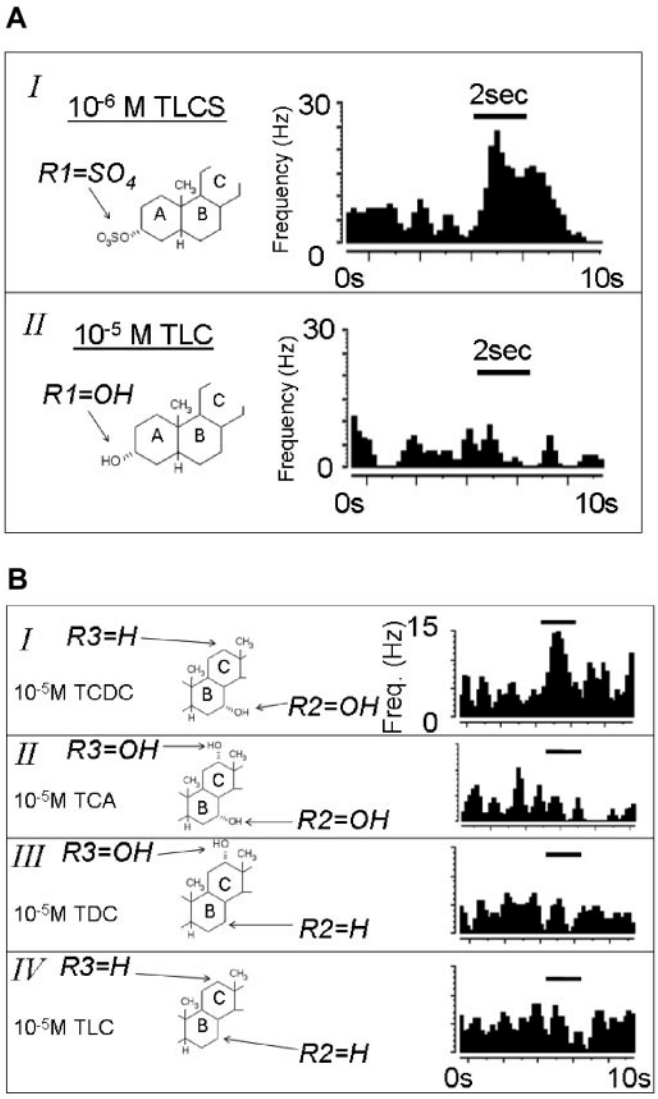



FIG. 6. Representative group T OB neurons. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. *A*: group T neuron (neuron 5 in Fig. 5) solely activated by TLCS (*I*). TLC (*II*), which differs from TLCS only by a –OH at R3 did not activate this neuron. Two second odor applications indicated by the black horizontal line in each rate histogram. *B*: single OB neuron (neuron 7 in Fig. 5) 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 *B, II–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.

17 neurons) were excited at 1 $\mu$M (Table 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 17 (65%) group N neurons responded to only one NBS; the remaining 6 neurons (35%) responded to two to three NBS (Fig. 7). Eight group N neurons were selectively excited by LCA (Figs. 7, neurons 1–8, and 8*A*). 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 responded to two to three NBS (Fig. 7). 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. 7 and 8*B*); 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 four NBS tested.

### *Group G OB neurons*

All 16 OB neurons classified as group G responded excitedly to at least one GBS, one TBS, and one NBS (Fig. 9). Excitatory thresholds for group G neurons ranged from 0.1 to 10 $\mu$M. Ninety-four percent (15 of 16 neurons) of group G neurons were excited by at least 1 of the 11 bile salts tested at 1 $\mu$M, whereas 19% (3 of 16 neurons) responded at 0.1 $\mu$M (Table 1). Only neuron 16 (Fig. 9) responded excitedly to a bile salt at concentration $\leq$0.01 M. For this neuron, excitatory responses were recorded to 0.1 nM to 10 $\mu$M CA. Group G neurons responded particularly well to GBS. For group G neurons, only GCDC elicited an excitatory response from all 16 neurons within this group, whereas 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 TBS (TLC, TLCS) and two NBS (LCA and DCA) lacking a 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. 9). 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-seven of the 51 recorded OB neurons (73%) were suppressed by $\geq$3 of the 11 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 = $SO_4$); Fig. 5, neurons 1–6; Fig. 6*A*]. TLC (R2 = H, R3 = H, R4 = $NHCH_2CH_2SO_3H$) also suppressed the spontaneous activity of neurons excited by LCA [(R2 = H, R3 = H and R4 = OH); Fig. 7, neurons 1–8]. Further, the spontaneous activity of neurons excited by LCA were generally suppressed by other NBS odorants (Fig. 7, 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.

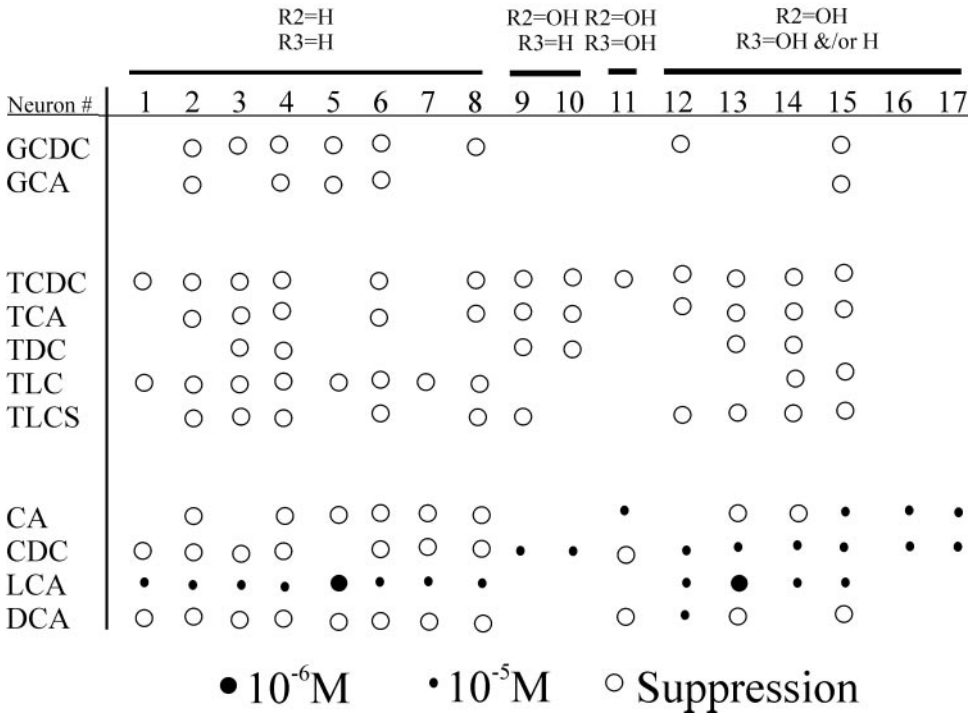



FIG. 7. Group N neurons are excited by only NBS. ●, excitatory responses. ○, inhibitory responses. No symbol, OB neuron activity to the odorant did not change significantly from prestimulus levels. The molecular features determined to be critical for eliciting an excitatory response are indicated above, excluding R4, which was an hydroxyl moiety.

## DISCUSSION

### *Bile salts in fishes and their function as olfactory cues*

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; Thwaits et al. 2006; Yeh and Hwang 2001; Zhang et al. 2001). Some marine species also produce cysteinolic acid-conjugated bile salts (Goto et al. 1996; Une et al. 1991); 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 blue catfish (*I. 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 (P. W. Sorensen, personal communication). Given that related fish species produce structurally similar bile salts, the exact ratio of particular bile salts within the bile may allow individuals to identify conspecifics by olfaction.

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 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 that 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 that 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 and Sorensen 2005; Fine et al. 2004; Li and Sorensen 1997; Li et al. 1995, 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 (Fine et al. 2004; Li et al. 1995, 2002; Sorensen et al. 2005) and select for streams containing populations of sea lamprey larvae that would indicate suitable spawning grounds (Bjerselius et al. 2000; Fine and Sorensen 2005; Polkinghorne et al. 2001; Vrieze and Sorensen 2001).

Electrophysiological data indicate that sea lampreys possess a complement of molecular ORs, 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 molecular ORs bind particular combinations of molecular features (-$SO_4$, -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

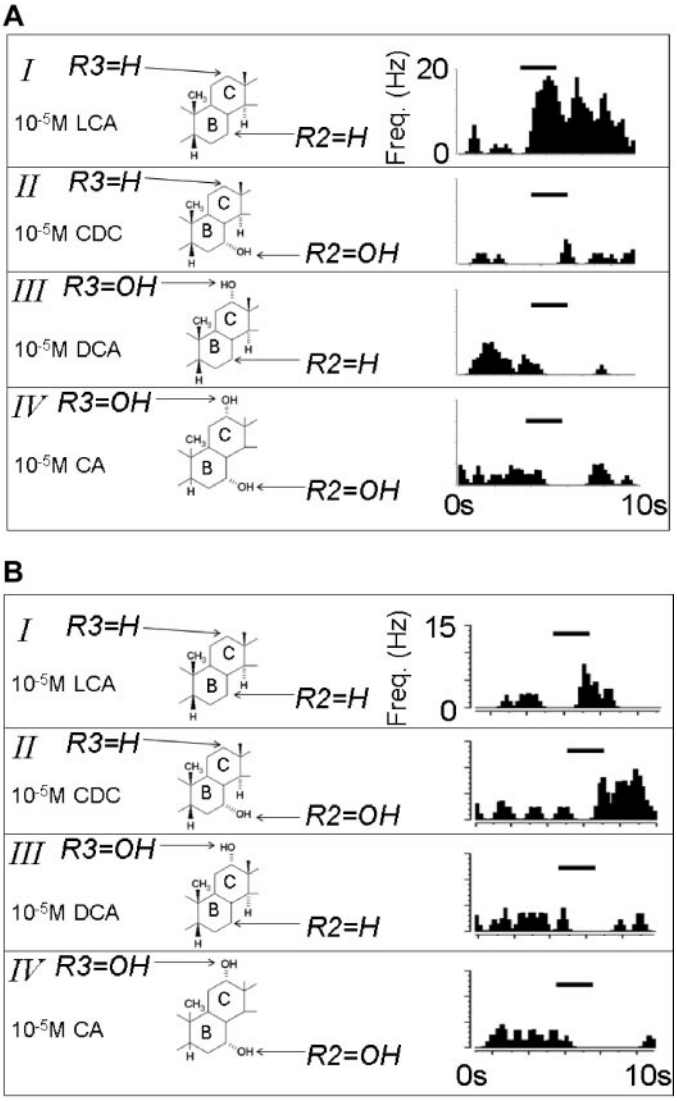



FIG. 8. Representative group N neurons. Rate histograms of single neuron activity are shown along with the structure of the relevant portions of each bile salt molecule. Two separate OB neurons are shown in *A* and *B*. *A*: OB neuron excited by LCA (*I*). Rate histograms for neuron activity in response to CDC (*II*), DCA (*III*), and CA (*IV*) are also shown to allow for comparison. Excitatory responses in this neuron required 2 molecular features along the steroid backbone (R2 = –H and R3 = –H) and 1 in the side-chain, R4 = –OH. *B*: 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 1 molecular feature of the steroid backbone (R3 = –H) and 1 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.

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 using 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 (Døving et al. 1980; Thommesen 1978; Zhang et al. 2001), trout (Hara et al. 1984; Laberge and Hara 2004), salmon (Hara and Zhang 1996), zebrafish (Friedrich and Korsching 1998; Michel and Derbidge 1997; Michel and Lubomudrov 1995), and channel catfish (Nikonov and Caprio 2001).

The studies discussed in the preceding text 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 composed 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 that 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 three 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, whereas 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 molecular ORs capable of discriminating TBS, GBS, and NBS, 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 nonreciprocal 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 molecular OR sites. Therefore it is likely that a large portion of the information regarding these pairs of bile salts is transmitted along the same neural pathways (OB and forebrain) in zebrafish. The present study found both OB neurons with EMRRs, which included both GCDC and GCA or TCDC and TCA, and OB neurons capable of discriminating these pairs of bile salts. Given that ORNs with like molecular ORs 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 nonreciprocal 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

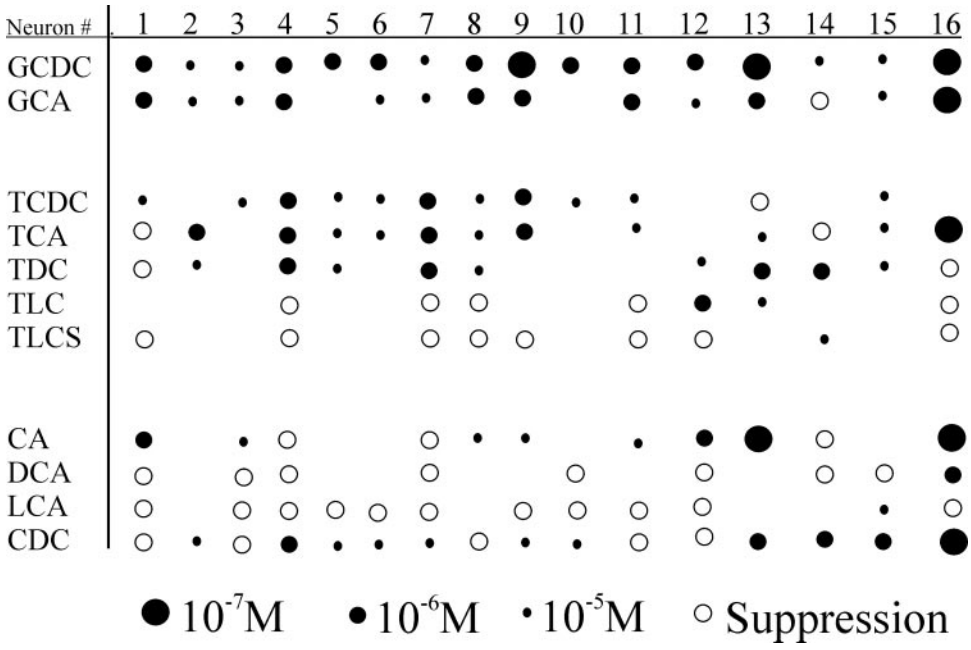



FIG. 9. Group G neurons are excited by TBS, GBS, and CBS. ●, excitatory responses. ○, inhibitory responses. No symbol, OB neuron activity to the odorant did not change significantly from prestimulus levels. For unit 16 only, excitatory responses were recorded to $10^{-10}$-$10^{-5}$M CA.

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 molecular OR sites for sulfonated bile salts. Li and Sorensen (1997) suggested that TLCS, lithocholic acid-3-sulfate (LCS) and glycolithocholic acid-3-sulfate (GLCS) likely bind to a common molecular OR 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 neuron selectivity determined electrophysiologically versus 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 GBS and TBS, with voltage-sensitive dyes loaded into ORNs (Friedrich and Korsching 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 (nonspecific). 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 groups G and T were intermingled with one another within the medial region of the catfish OB. Intermingling of OB neurons possessing EMRRs for both GCA/TCA (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.

### *Specificity of catfish OB neurons versus that 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. Because the EMRR of a bile salt molecular OR has not yet been characterized, no direct comparison between our electrophysiological results and molecular OR 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 (Kawamata et al. 2003; Maruyama et al. 2002). TGR5/BG37 and fish molecular ORs are structurally similar (7-transmembrane domain) and both TGR5/BG37 (Kawamata et al. 2003; Maruyama et al. 2002) and fish molecular ORs (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 and nonconjugation at C24 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 with group G.

## GRANTS

This work was supported by the National Science Foundation Grant IBN-0314970 and the National Institute of Deafness and Other Communication Disorders Grant DC-03792.

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