

Odorant Specificity of Single Olfactory Bulb Neurons to Amino Acids in the Channel Catfish

Alexander A. Nikonov and John Caprio

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

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Nikonov, Alexander A. and John Caprio. Odorant specificity of single olfactory bulb neurons to amino acids in the channel catfish. *J Neurophysiol* 92: 123–134, 2004. First published February 11, 2004; 10.1152/jn.00023.2004. Odorant specificity to L- α -amino acids was determined for 245 olfactory bulb (OB) neurons recorded from 121 channel catfish. The initial tests included 4 amino acids representing acidic [monosodium glutamate (Glu)], basic [arginine (Arg)], and neutral [possessing short: alanine (Ala) and long: methionine (Met) side chains] amino acids that were previously indicated to bind to independent olfactory receptor sites. Ninety-one (37%) units (Group I) tested at 1, 10, and 100 μ M showed high selectivity and were excited by only one of the 4 amino acids. Odorant specificity for the vast majority of Group I units did not change over the 3 s of response time analyzed. A total of 154 OB units (63%) (Group II) were excited by a second amino acid, but only at $\geq 10\times$ odorant concentration. An additional 69 Group I units were tested with related amino acids and derivatives from 10^{-9} to 10^{-5} M to determine their excitatory odorant thresholds and selectivities. Two groups of units originally selective for Met were evident: those most sensitive to neutral amino acids having branched and linear side chains, respectively. OB units originally selective for Ala responded at low concentration to other similar amino acids. Units originally selective for Arg were excited at low concentration by amino acids possessing in their side chains at least 3 methylene groups and a terminal amide or guanidinium group. The specificities of the OB units determined electrophysiologically are sufficient to account for many of the previous results of behavioral discrimination of amino acids in this and related species.

INTRODUCTION

Olfaction begins with the detection of odorants by 7 transmembrane G-protein-coupled olfactory receptors (ORs) located in the apical membranes of olfactory receptor neurons (ORNs) (Buck and Axel 1991; Clyne et al. 1999; Ngai et al. 1993b; Vosshall et al. 1999). Each ORN expresses one or a few ORs from large gene families (Chess et al. 1994; Malnic et al. 1999; Ressler et al. 1993; Vassar et al. 1993). ORNs that express a given OR gene are located within one of 4 broad, nonoverlapping zones in rodents (Ressler et al. 1993; Sullivan and Dryer 1996; Vassar et al. 1993); 3 “fuzzy” expression zones in zebrafish (Weth et al. 1996); or a single zone in channel catfish (Ngai et al. 1993a). Each OR binds multiple odorants, and multiple ORs participate in the detection of each odorant (Kajiya et al. 2001; Malnic et al. 1999). Olfactory transduction involves second-messenger pathways of which the c-AMP pathway has been most studied in tetrapods (Schild and Restrepo 1998); the IP₃ pathway, however, appears to be important in olfaction in aquatic organisms, such as lobster

(Hatt and Ache 1994) and fish (Bruch 1996; Speca et al. 1999). The axons of ORNs expressing like ORs converge onto specific target glomeruli in vertebrates in the olfactory bulb (OB) (Mombaerts et al. 1996; Ressler et al. 1994; Strotmann et al. 2000; Vassar et al. 1994) where they synapse onto apical dendrites of mitral/tufted relay neurons, forming a functional map relating general chemical features of odorant structure to specific glomerular fields (Xu et al. 2000). The unique combination of glomeruli activated in response to an odor is thus thought to define (i.e., code for) the odor. Thus an individual glomerular module (a glomerulus and its associated neurons) is a molecular feature detector where different glomerular modules are “tuned” to odorants, somewhat like ORs, but modified by intraglomerular circuitry (Sachse and Galizia 2002; Yokoi et al. 1995).

A key to understanding any sensory system for a specific species is deciphering what the biologically relevant stimuli are. Whereas olfactory stimuli tested for mammals are rather broad, with many stimuli having questionable biological significance, the olfactory system of fish responds to fewer odorants with a known behavioral context. Amino acids, which constitute a major class of biologically relevant odorant stimuli, are used as feeding cues by teleosts. Behavioral studies in both intact and anosmic catfish showed conclusively that, although fish taste and smell L- α -amino acids, a functioning olfactory system is required for the learned search images for specific amino acids (Valenticic et al. 1994). For example, although separate populations of peripheral fibers process L-alanine (Ala) and L-arginine (Arg) taste information in the channel catfish (Kohbara et al. 1992), anosmic specimens were unable to discriminate even between these 2 amino acids in a behavioral conditioning paradigm. These results indicate that olfactory receptor neurons (ORNs) and some proportion of ascending neurons within the olfactory system must respond differentially to amino acids to allow for their learned behavioral discrimination. However, the neural basis for this discriminatory behavior is presently unknown. Although many electrophysiological studies in fishes tested amino acids as odorant stimuli, few of these reports focused on the responses of single neurons to these stimuli at either the receptor cell (Kang and Caprio 1995c, 1997; Sato and Suzuki 2001) or bulbar (Kang and Caprio 1995a,b; MacLeod 1976; Meredith 1981; Meredith and Moulton 1978) neural levels. These previous studies, however, did not provide any evidence for the existence of neuron types within the olfactory pathway that were excited by specific amino acids. Recent calcium-imaging (Friedrich and Kor-

Address for reprint requests and other correspondence: J. Caprio, Dept. of Biological Sciences, LSB Rm 202, Louisiana State University, Baton Rouge, LA 70803 (E-mail: jcap@lsu.edu).

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sching 1997; Fuss and Korsching 2001) and voltage-imaging (Friedrich and Korsching 1998) studies of ORN nerve terminals within the OB of zebrafish did reveal OB regions responsive to particular types of amino acids, but responses of individual cells were not detected.

Single OB neurons can be recorded in an intact preparation over longer average times than single ORNs (Kang and Caprio 1995a,b, 1997). The present in vivo electrophysiological study of the responses of single OB neurons in the channel catfish provides evidence for different OB unit types based on their specificities to structurally different amino acids at concentrations slightly above threshold where OB units are generally most selective (Friedrich and Korsching 1997). It is likely that catfish and other teleosts detect and discriminate among amino acids during their appetitive food search behavior at similar concentrations as tested in this investigation.

METHODS

Experimental animals

Channel catfish, *Ictalurus punctatus* (15–20 cm total length), obtained from a local hatchery, were maintained in floating cages held in ponds at the Louisiana State University Aquaculture Center facility. The fish were fed weekly with floating commercial fish chow. Each week catfish were transferred to an aerated, 250-liter polyethylene aquarium filled with charcoal-filtered city tap water (CFTW) at the Louisiana State University Animal Care Facility and maintained on a 12:12 light/dark regime. The temperature was held above 27°C during the spring and summer and below 20°C during the fall and winter to inhibit growth of the pathogenic bacterium, *Edwardsiella ictaluri*, which causes enteric septicemia and destroys chemosensory epithelia (Morrison and Plumb 1994). The fish were used experimentally within a 1-wk holding time and were not fed during this period.

Animal immobilization and anesthesia

The preparation of the animals was the same as that previously described (Nikonov and Caprio 2001). Each catfish was initially immobilized with an intramuscular injection of the neuromuscular blocking agent Flaxedil (gallamine triethiodide, 0.03 mg/100 g). During the experiments, additional injections were applied as needed by a hypodermic needle embedded in the flank musculature. The immobilized fish was wrapped in a wet Kim-Wipe, placed into a Plexiglas container and stabilized using a pair of orbital ridge clamps. The gills were irrigated using an orally inserted glass tube supplying a constant flow of aerated, charcoal-filtered city tap water that initially contained the anesthetic, 50 mg/l MS-222 (ethyl-*m*-aminobenzoate methane sulfonic acid). Surgical wounds were also bathed with 3% tetracaine. Once surgery was completed, the gill irrigation water was replaced with CFTW not containing MS-222; however, applications of the tetracaine were continued.

Surgical preparation

Access to the olfactory organ was achieved by removing skin and connective tissue between the incurrent and excurrent nares, superficial to the olfactory organ. The pedunculated OB was also exposed by removing an approximate 1-cm section of skin and subcutaneous fat at the midline of the fish caudal to the nasal capsule. After the removal of the underlying bone and cartilage, suction was applied to remove adipose tissue from the cranial cavity and the open space was filled with fresh-water teleost Ringer solution.

Odorant stimuli and delivery

The odorant stimuli (amino acids) were obtained commercially (Sigma Chemical) and were the purest available. Stock solutions

(10^{-3} M) of representatives of 4 different classes of amino acids [i.e., acidic, basic, neutral with long side chains (LCN), and neutral with short side chains (SCN)], previously shown (Kang and Caprio 1997) to be potent stimuli to olfactory receptor neurons of channel catfish, were prepared weekly in CFTW. Log-step dilutions in CFTW of the amino acid odorants were made daily. Stimulus delivery was by a "gravity-feed" system using a spring-loaded valve (Model 5301, Rheodyne, Cotati, CA) driven by a pneumatic actuator (Model 5300) at 40 psi (Nikonov et al. 2002; Sveinson and Hara 2000). Stimulus solutions and the CFTW used to bathe the olfactory mucosa between stimuli were delivered through separate Teflon tubes (0.8 mm diameter) at a rate of 6–8 ml/min. The olfactory cavity was continuously perfused with CFTW to 1) facilitate stimulus delivery, 2) protect the mucosa from desiccation, 3) avoid the introduction of mechanical artifacts associated with stimulus presentation, and 4) thoroughly rinse the olfactory cavity between stimuli (3–5 min interstimulus intervals). A foot switch connected to an electronic timer (Model 645, GraLab Instruments Division, Dimco-Gray, Centerville, OH) triggered the valve to introduce the odorants for a 3-s stimulus duration, without a change in either pressure or temperature and with minimal dilution.

Recording techniques

THE ELECTROOLFACTOGRAM (EOG). The underwater electroolfactogram (EOG) is an odorant-induced, slow negative potential measured in the water immediately above the olfactory mucosa, which reflects summated olfactory receptor generator potentials (Ottoson 1971). The EOG was recorded in vivo with calomel electrodes by Ringer-agar-filled capillary pipettes as reported previously (Silver et al. 1976). The EOG signal was amplified (Grass P-18 DC amplifier), printed on a chart recorder, digitized, and stored on a video channel of a high-fidelity VCR recorder. The EOG signal served as an indicator of both the viability of the preparation and the response onset to the tested odorants.

OB UNIT RECORDINGS. Unit/few unit activity (range 250–1,000 μ V peak-to-peak amplitude) was recorded extracellularly from the amino acid, mediolateral zone within the OB (Nikonov and Caprio 2001). The electrode, a low-impedance (2–5 M Ω) platinum and gold-plated, metal-filled, glass micropipette (glass tip, 2 μ m; ball diameter, 3–4 μ m), was mounted on a hydraulic microdrive attached to a stereotaxic micromanipulator and advanced vertically downward from the dorsal surface of the olfactory bulb. The majority of recordings of bulbar neurons was obtained at primarily 2 ranges of depth, 150–400 and 700–1,000 μ m, where the cell bodies and dendrites of mitral cells are located in catfish (J. Kang and J. Caprio, unpublished observations). Odor application began once a spontaneously active unit was encountered and was clearly isolated by fine-positioning the recording electrode by the remote fluid-filled microdrive. The neural activity was amplified (band-pass 30–10,000 Hz; Grass Instruments P511k), observed with an oscilloscope and stored on an audio channel of a high-fidelity VCR.

DATA ACQUISITION AND ANALYSIS. All recorded data from both the olfactory lamellae and olfactory bulb were digitized at 32 kHz, analyzed off-line by Discovery software (Brainwave Systems Discovery package Version 5.0 with Autocut, DataWave Technologies, Longmont, CO), and printed. Some of the waveform parameters that were used by the software to identify and discriminate extracellularly recorded action potentials were peak amplitude, valley amplitude, spike height, spike width, spike time, and time between spikes. Spike events, EOG signals, and experimental parameters (i.e., beginning of a recording period, onset of stimulation, and end of the recording period) were time-stamped with a 32-bit 100- μ s resolution value and saved in a data file. The BrainWave data files were displayed on a computer screen and viewed by Neuroexplorer (Nex Technologies, Lexington, MA) software.

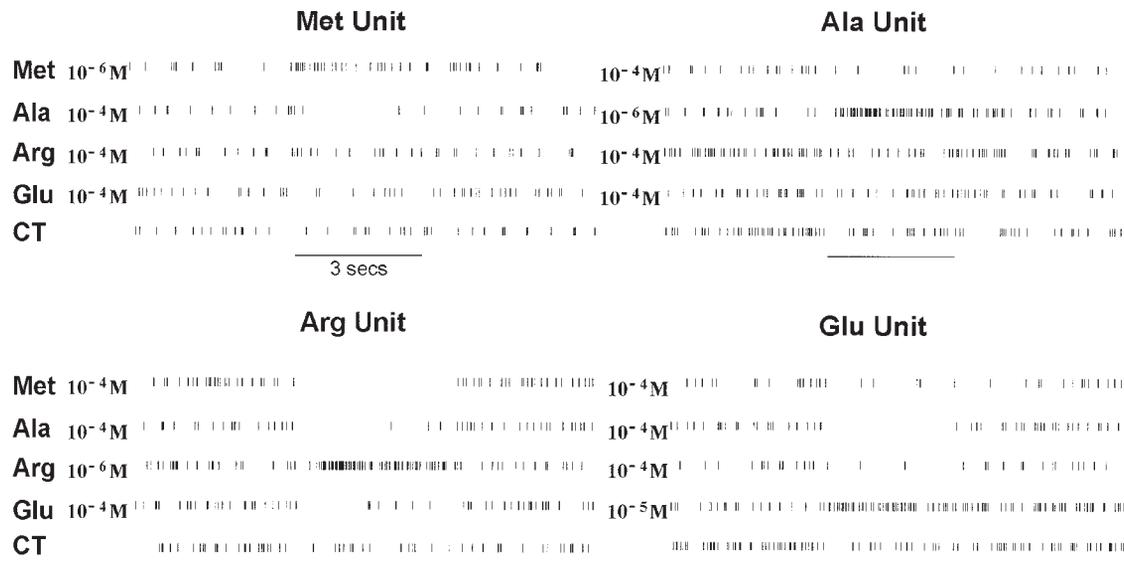


FIG. 1. Responses of representative Met, Ala, Arg, and Glu Group I olfactory bulb (OB) units to odorants representing 4 major types [long chain neutral (LCN), short chain neutral (SCN), basic amino acid (B), and acidic amino acid (A), respectively] of amino acids and to control water (CT). Tested concentrations were from 10^{-6} to 10^{-4} M. Concentration listed adjacent to each record of action potentials indicates the lowest concentration that resulted in an excitatory response for that unit. Odorant onset and duration (3 s) are indicated by the line below each *control trace*.

Responses of single olfactory bulb 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 1995a). The ITSA compares statistically the number of action potentials occurring within successive 250-ms time bins for 3 s before and after the initial onset of the odor-induced EOG. In a subset of experiments, the ITSA was used to analyze response times that encompassed <3 s (i.e., the first and third seconds, respectively) of the response period.

RESULTS

Unit selectivity to amino acid type: the excitatory molecular response range

The present study analyzed only excitatory responses because it is the excitatory response that drives the response of postsynaptic neurons at the next ascending level of the olfactory system in the cerebral lobes.

GROUP I UNITS: PRIMARY SEARCH STRATEGY. A total of 327 OB units from 208 dorsal and 119 ventral OB sites in a total of 103 channel catfish were each tested with 10^{-6} – 10^{-4} M L-isomers of a representative acidic amino acid (A) [sodium glutamate (Glu)], a representative basic amino acid (B) [arginine (Arg)], a representative neutral amino acid with a short side chain (SCN) [alanine (Ala)], and a representative neutral amino acid with a long side chain (LCN) [methionine (Met)]. These odorants were selected because previous electrophysiological cross-

adaptation (Caprio and Byrd Jr 1984) and biochemical binding (Bruch and Rulli 1988) studies in this species indicated independent molecular olfactory receptors for these types of amino acids. A total of 245 (75%) of these units responded excitedly to at least one of the 4 representative amino acid types tested between 10^{-6} and 10^{-4} M. Of the units that were excited, 91 (37%) were highly specific and responded to only one of the 4 types of test amino acids (Group I Units) (Fig. 1; Table 1). The majority (86%) of these units responded to $\geq 10^{-6}$ M amino acid; the remaining units responded at $\geq 10^{-5}$ M. None of these highly selective units was excited by any of the other representative types of amino acids at odorant concentrations up to and including 10^{-4} M. The majority of these highly selective units were excited by either Met ($n = 31$; 34%) or Arg ($n = 28$; 31%); units excited by either Ala ($n = 19$; 21%) or Glu ($n = 13$; 14%) were fewer.

Analysis of response time. We addressed the question of whether our classification of response type based on an analysis of 3 s of response time would be significantly altered by analyzing different portions of the response to 3-s stimulus applications. We therefore analyzed the odorant-induced responses of single Group I bulbar neurons to amino acids during the first and third seconds of the responses to determine whether there was evidence of significant changes in unit selectivity (Table 2). Of 78 Group I units analyzed (i.e., those that were originally determined to be selectively responsive to only Met, Ala, Arg, or Glu over 3 s of response), 81 and 85%

TABLE 1. *Group I units*

	Met Units ($n = 31$)	Ala Units ($n = 19$)	Arg Units ($n = 28$)	Glu Units ($n = 13$)
Excitatory response (10^{-6} M)	Met 74% ($n = 23$)	Ala 100% ($n = 19$)	Arg 86% ($n = 24$)	Glu 92% ($n = 12$)
No excitatory response (10^{-6} – 10^{-4} M)	Ala Arg Glu	Met Arg Glu	Met Ala Glu	Met Ala Arg

TABLE 2. Specificity of OB neurons over response time (RT)

Unit Type	Number of Units Classified Based on 3-s RT	Number of Units Classified Based on 1st Second of Response (% 3-s RT)	Number of Units Classified during 3rd Second of Response (% 3-s RT)
Met	23	17 (74)	21 (91)
Ala	19	16 (84)	15 (79)
Arg	24	21 (88)	20 (83)
Glu	12	9 (75)	10 (83)

were similarly classified when analyzing the first and third seconds of the responses, respectively.

GROUP II UNITS: PRIMARY SEARCH STRATEGY. The remaining 154 (63%) of the 245 units (i.e., Group II) (Table 3) tested had a broader specificity than that of the Group I units, but particular types were still rather specific in their responses, given that the sensitivities to the amino acid types were not randomly distributed (i.e., each unit type was not responsive to other particular types of amino acids). Similar to the Group I units, the more numerous of the Group II units were those excited by $\geq 10^{-6}$ M Met ($n = 83$; 54%) or $\geq 10^{-6}$ M Arg ($n = 46$; 30%); units excited by $\geq 10^{-6}$ M Ala ($n = 21$; 14%) or Glu ($n = 4$; 2%) were fewer. The majority (70 of 83; 84%) of the Group II units were excited by $\geq 10^{-6}$ M amino acid; the remaining units responded to $\geq 10^{-5}$ M. With only one exception out of a total of 50 OB units analyzed, all of the Group II OB units that were most sensitive to Arg and Glu, respectively, were highly selective in their responses $\leq 10^{-4}$ M amino acid, which is the initial concentration that Glu stimulated the Arg units and Met stimulated the Glu units. The majority (93%; 77 of 83) of the Group II units having lowest threshold to methionine, a neutral amino acid possessing a long side chain, were also excited by alanine, a neutral amino acid with a short side chain, but at a 10-fold higher stimulus concentration. The converse, however, did not occur, in that units with lowest thresholds to Ala were not excited by Met. Nevertheless, the Met unit group did show high specificity to neutral amino acids because neither 10^{-4} M Arg nor Glu excited any of the 83 Met units tested. The Ala units, however, were the least specifically tuned of the Group II OB neurons because 86% were also excited by 10^{-4} M Arg and 71% by 10^{-4} M Glu; only Met (an LCN) failed to stimulate the Group II Ala units at concentrations $\leq 10^{-4}$ M. Although only 7% (17 of 245) of the OB neurons analyzed (including Groups I and II) had the lowest thresholds to Glu, this amino acid at high stimulus concentrations stimulated the vast majority (90%; 60 of 67 units) of 2 of the 3 other types of Group II units.

TABLE 3. Group II units

	Met Units ($n = 83$)	Ala Units ($n = 21$)	Arg Units ($n = 46$)	Glu Units ($n = 4$)
Excitatory response				
10^{-6} – 10^{-4} M	Met 93% (77)	Ala 81% (17)	Arg 89% (41)	Glu 75% (3)
	10^{-6} M	10^{-6} M	10^{-6} M	10^{-6} M
10^{-5} M	Ala 93% (77)	Glu 29% (6)	—	—
10^{-4} M	Ala 95% (79)	Glu 71% (15)	Glu 98% (45)	Met 100% (4)
		Arg 86% (18)		
No excitatory response	Arg	Met	Met	Arg
10^{-6} – 10^{-4} M	Glu		Ala	Ala

GROUP I UNITS: EXPANDED SEARCH STRATEGY. Because the Met (LCN), Ala (SCN), and Arg (B) units were highly selective for their amino acid type, we further explored in 18 additional catfish their amino acid specificities to additional amino acids and derivatives whose chemical structures resembled Met, Arg, and Ala, respectively. A total of 69 additional spontaneously active Group I neurons were located that were selectively excited by either 10^{-6} M Met ($n = 31$), Arg ($n = 26$), or Ala ($n = 12$) of the 4 amino acids tested from 10^{-6} to 10^{-4} M (units with lowest threshold to glutamate were too few and were not tested further). These units were then tested with 10^{-9} – 10^{-5} M concentrations of Met, Ala, and Arg, respectively. The OB units: 1) previously selectively excited by Met were tested with 8 related compounds to Met: L-valine (Val); L-leucine (Leu), L-isoleucine (iLeu), L-norvaline (nVal), L-norleucine (nLeu), S-ethyl-L-cysteine (EtCys), L-ethionine (Et), and L-methionine sulfoxide (MetSul); 2) units previously selectively excited by Ala were tested with 4 related amino acids to Ala: glycine (Gly), L-serine (Ser), L- α -aminobutyric acid (Abu), and L-threonine (Thr); and 3) units previously excited by Arg were tested with 8 related compounds to Arg: L-lysine (Lys), L-homoarginine (HArg), L-ornithine (Orn), L- α -amino- β -gaunidino propionic acid (AGPA, a truncated Arg side chain containing 2 fewer methylene groups), L-citrulline (Cit), aminoguanidinium (AG, related to the terminal structure of the Arg side chain), putrescine (Put, a decarboxylated Orn), and cadaverine (Cad, a decarboxylated Lys). The classification of the type of amino acid unit identified in this report was based on threshold estimation.

Selectivity of Group I Met units. Although there was some overlap in excitatory responses of single OB units to the tested neutral amino acids, 2 general groups emerged consisting of units most responsive to amino acids either with long, linear side chains or with branched side chains (Figs. 2 and 3). This classification of the type of LCN unit identified was based on the particular amino acids [i.e., having linear (ILCN) or branched (bCN) side chains] that excited the cell at the lowest odorant concentration and the number of such cases. These 31 units were separated into 3 general categories: 1) 13 units whose excitatory thresholds were lowest to ILCNs, possessing side chains consisting of 3–4 methylene groups (i.e., nVal, nLeu, and/or Met) (Fig. 3A); 2) 16 units whose excitatory thresholds were lowest to bCNs (Val, Leu, and/or iLeu) (Fig. 3B); and 3) 2 units whose excitatory thresholds were similar to both ILCNs and bCNs (Fig. 3C). A further indication of this dichotomy is that the OB units with lowest thresholds to ILCNs were generally also excited by the 3 amino acid analogs (Et-Cys, Et, and MetSul) tested that possessed long, linear side chains (Fig. 3), whereas few of the OB units that were desig-

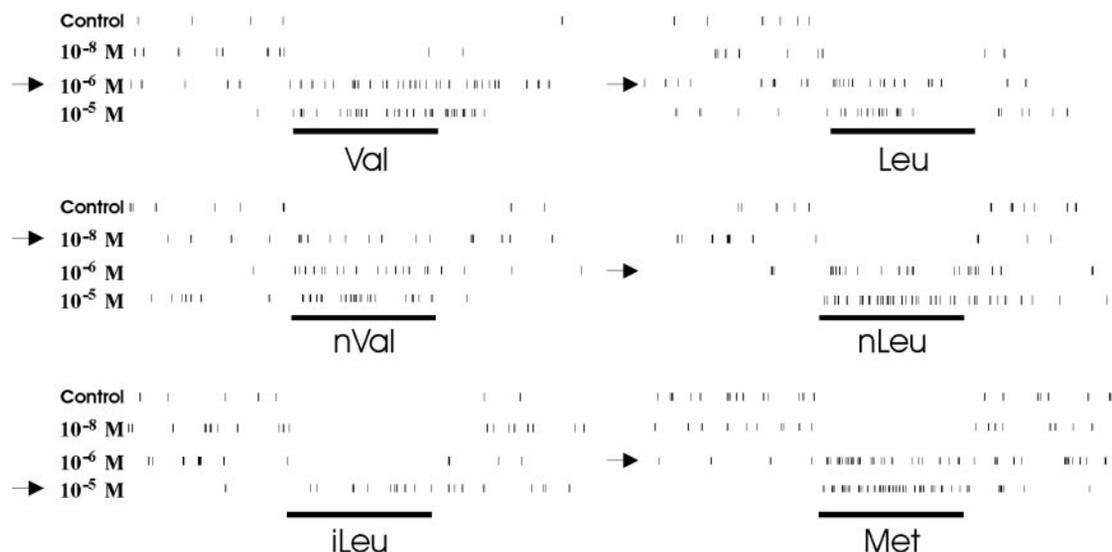


FIG. 2. Responses of a representative OB unit (cell 6 in Fig. 3) to neutral amino acids with branched (bCNs: Val; Leu; iLeu) and linear (ILCNs: nVal; nLeu; Met) side chains. Odorant concentrations are listed adjacent to each record; arrow indicates the lowest odorant concentrations that resulted in an excitatory response for that unit. Odorant onset and duration (3 s) are indicated by the line below each *control trace*.

nated as having lowest thresholds to bCNs were excited by these latter compounds. Interestingly, nVal, the ILCN possessing the shortest linear side chain consisting of 3 methylene groups, was treated by the majority of OB units tested as both an ILCN and a bCN because 12 of the 13 ILCN units and 10 of the 16 bCN units were excited by nVal at the lowest threshold for the amino acids tested for those units. OB units were also evident that were most sensitive to different amino acids within each category. For example, of the ILCNs, unit 6 had the lowest threshold to nVal, whereas of the bCNs, unit 26 had the lowest threshold to Val, and units 27 and 28 had the lowest thresholds to Leu (Fig. 3). No units were identified whose excitatory thresholds were lowest to any of the 3 Met derivatives, EtCys, Et, or MetSul. Further, over the concentrations tested, 36% (10/28) of the bulb units were not excited by EtCys, 61% (17/28) were not excited by Et, and 57% (16/31) were not excited by MetSul. Importantly, all 31 of these OB units were excited by $\geq 10^{-5}$ M Met, thereby justifying the selection of Met as a representative LCN amino acid in our original classification of unit types.

Selectivity of Group I Ala units. Four different odorants related to Ala were tested on 12 additional Group I bulbar units that were selectively excited by Ala (Fig. 4). Three (units 5–7) of the 12 units were highly sensitive and were excited by 10^{-8} M Ala; the remaining 9 units responded excitedly to 10^{-7} – 10^{-6} M Ala. Seven units (1–7) responded with highest sensitivity to Ala, whereas the threshold concentration to Ala for all 5 of the remaining units was shared with Ser, a hydroxylated alanine. Only one of the 12 units was excited by Gly. Importantly, all 12 of these OB units were excited by $\geq 10^{-6}$ M Ala, thereby justifying the selection of Ala as a representative SCN amino acid in our original classification of unit types.

Selectivity of Group I Arg units. Eight different odorants related to Arg, Lys, and other basic amino acids were tested on 26 additional Group I bulbar units that were selectively excited by Arg (Figs. 5 and 6). Nineteen (73%) of the 26 OB units tested were highly sensitive and were excited by 10^{-9} or 10^{-8} M basic amino acid (Fig. 6). OB units were identified that

responded at lowest threshold to as few as a single basic amino acid (units 1–4) to those units having the lowest threshold to 5 different basic amino acids tested (units 25 and 26) (Fig. 6). In comparing the estimated thresholds of the 2 common basic amino acids, Lys and Arg, 7 OB units (Fig. 6, units 1, 5–10) exhibited a lower excitatory threshold to Lys than to Arg; 3 units (Fig. 6, units 11–13) were more sensitive to Arg; and the remaining 10 units had similar excitatory thresholds to both Lys and Arg. Importantly, of these 26 OB units tested, 20 (77%) were excited by 10^{-7} – 10^{-6} M Arg and the remaining 6 by 10^{-5} M Arg, thereby justifying the selection of Arg as a representative basic amino acid in our original classification of unit types. Of the 7 basic amino acids tested in addition to Lys and Arg, HArg and ornithine were also relatively potent stimuli. Thresholds to HArg across the units tested were often similar to that to Lys or to Arg; however, in 2 cases (Fig. 6, units 14 and 15) the threshold to HArg was ≥ 10 -fold lower than to either Lys or Arg. In 3 cases, thresholds were lowest to Orn (Fig. 6, units 2–4), whereas for most cases ORN thresholds were similar to those to Lys and/or Arg. None of the 26 units was excited by low (10^{-9} – 10^{-8} M) concentrations of Cit, Put, Cad, or AG. Further, of 26 OB units tested, only 7 (27%) responded excitedly to Cit, 3 (12%) to Put, 2 (8%) to Cad, and 1 (4%) to AG, but all only at 10^{-5} M.

DISCUSSION

Organization of the teleost OB

Although the general anatomical organization of the olfactory system is similar across the vertebrates, the structure of neural connections within the olfactory bulb is different between fish and mammals (for a review see Dryer and Graziadei 1994). In contrast to mammals where single mitral cells project a single primary dendrite to a single large (50–200 μm in diameter), well-defined glomerulus, mitral cells in fish project primary dendrites to several different, small (10–20 μm in diameter) and less well-defined glomeruli (Mori 1995; Riddle

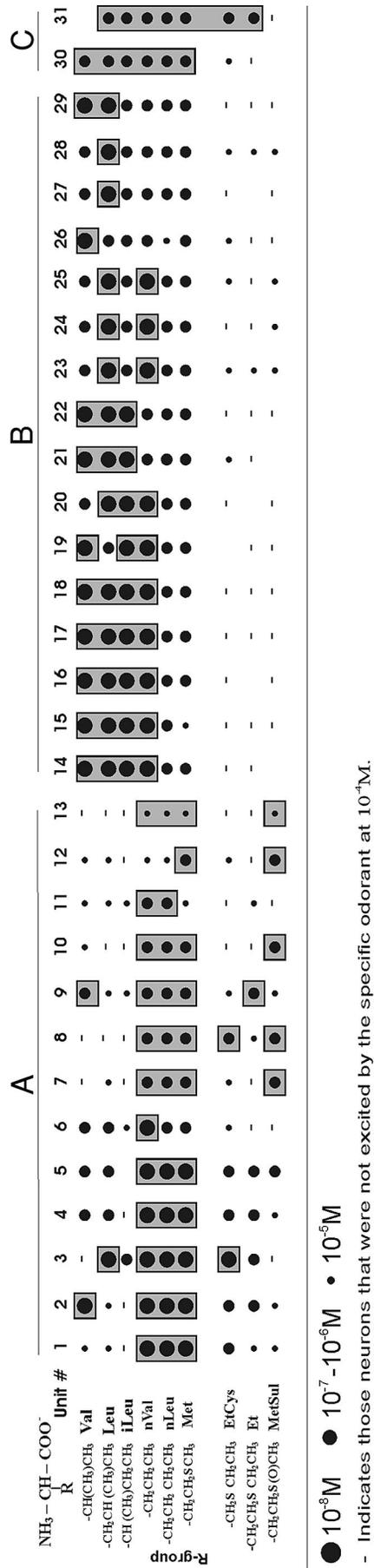


FIG. 3. Electrophysiologically derived excitatory thresholds of 31 OB Group I neurons to bCNS and ILCNs. Dots within the shaded boxes indicate both the concentrations and specific odorant(s) that resulted in an excitatory response at the lowest test concentration tested for each OB unit analyzed. A: indicates the 13 OB units with lowest thresholds to ILCNs (units 1–13). B: indicates the 16 OB units with lowest thresholds to bCNS (units 14–29). C: indicates 2 units whose excitatory thresholds were similar across the majority of odorants tested (units 30 and 31). – indicates those neurons that were not excited by the particular odorant tested at 10^{-4} M.

and Oakley 1992; Satou et al. 1983). The dendritic field of a single mitral cell in fish can extend for more than 300–400 μm from the soma (Kosaka and Hama 1982b; Oka 1983) and terminate in either discrete tufts or in diffuse, brushlike endings (Kosaka and Hama 1982b; Riddle and Oakley 1992). However, in spite of this extensive dendritic field, the neuronal activities on one side of the olfactory bulb are not influenced much by those in the opposite side (Satou 1990), suggesting that there is little crossing of dendritic fields to opposite sides of the bulb. The recent evidence for an odotopy within OB of the channel catfish (Nikonov and Caprio 2001) supports the functional isolation between right and left OB. In other aspects, mitral cells within fish lack basal dendrites and thus lateral interactions between mitral cells occur at the bases of their primary dendrites (Ichikawa 1976; Kosaka and Hama 1982a; Oka 1983). Further, axons of fish mitral cells arise not only from cell bodies, but from thick dendrites (Dryer and Graziadei 1994). Ruffed neurons, a cell type not observed in tetrapods, is also located within the mitral cell layer in teleosts; these cells synapse with granule cells, but not with ORNs (Kosaka and Hama 1979, 1981). Tufted and periglomerular cells are apparently lacking in the teleost OB (Dryer and Graziadei 1994; Satou 1990).

Numerous physiological reports exist concerning odorant specificity within the tetrapod (largely amphibian and mammalian) OB; however, few recent studies addressed the specific selectivities of OB units to biologically relevant odorants in teleosts, the largest class of extant vertebrates (Hamdani and Doving 2003; Kang and Caprio 1995a,b; Nikonov and Caprio 2001; Zippel et al. 2000). Prior studies in the channel catfish (Kang and Caprio 1995a,b) documented some basic response characteristics of OB neurons to amino acid odor stimulation. However, in these earlier experiments, the amino acid odorant concentrations applied were generally rather high and the chemotopy of the OB was unknown. These earlier reports also documented that OB units in the channel catfish could respond either excitedly or suppressively to amino acids over a wide range of stimulus concentrations and that the responses of single OB units did not change from excitation to suppression, or vice versa, over time. The present report reinvestigated the amino acid selectivities of single OB neurons in the channel catfish at generally lower stimulus concentrations than previously tested and with a larger number of amino acid stimuli. As indicated in a previous investigation (Kang and Caprio 1995a), the majority of OB units recorded in the present study were likely mitral cells based on the distribution of recording depths in the OB being compatible with the locations of the majority of cell bodies of mitral cells identified previously in histological section, and the large and relative constant amplitudes of the action potentials suggestive of being elicited by the large mitral cells. Although some percentage of the recordings in the present report were possibly from interneurons and ruffed neurons, the recorded selectivities recorded still provide information as to odorant selectivity within ORN terminal fields (Kauer and Cinelli 1993).

Previous studies indicated that mitral cells in mammals located within single glomeruli and those in neighboring glomeruli have related odorant selectivities, but those far apart often have quite different selectivities (Buonviso and Chaput 1990; Johnson et al. 1999; Meister and Bonhoeffer 2001; Mori and Yoshihara 1995; Tsuboi et al. 1999; Uchida et al. 2000). In

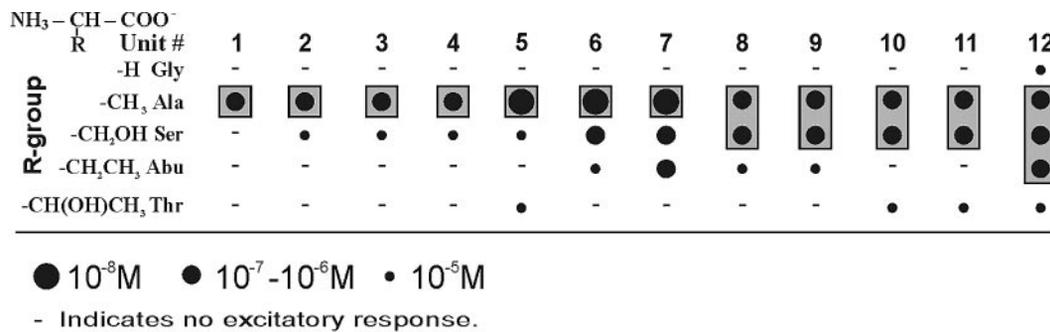


FIG. 4. Electrophysiologically derived excitatory thresholds of 12 OB Group I neurons to SCNs. Dots within the shaded boxes indicate both the concentrations and specific odorant(s) that resulted in an excitatory response at the lowest test concentration for each OB unit analyzed. - indicates those neurons that were not excited by the particular odorant tested at 10⁻⁴ M.

the present report, OB units in the channel catfish that were selective for a specific amino acid were located both close together (~250 μ m) and up to about 1 mm apart, at far extremes within the amino acid-responsive bulbar zone (Nikonov and Caprio 2001). Thus the odotopy present within the teleost OB appears not to be as precisely organized as that reported for mammals.

Selection of amino acid odorants

The study of the physiology of the olfactory system in any organism is immediately biased by the choice of odorants tested. Choosing appropriate biologically relevant stimuli is essential for a better understanding of the natural relevance of any measured activity. For fish, amino acids that are water soluble and released into the water column are well known, potent odorants important in the feeding behavior of aquatic organisms (Sorensen and Caprio 1998). A prior study documented the exquisite ability of catfish to discriminate amino acids behaviorally through olfaction (Valenticic et al. 1994). An investigation of the odorant specificity of individual neurons within the OB of a teleost to representative amino acids is likely to provide clues as to how the nervous system accom-

plishes this task for any vertebrate. Thus the present electrophysiological study examines the amino acid specificities of single neurons residing within the amino acid chemotopic zone of the OB in the channel catfish (Nikonov and Caprio 2001), a representative teleost species and model organism for olfactory research. The channel catfish is presently the only vertebrate species in which there is a clear correlation between the morphological type of ORN, the specific molecular transduction system, the type of biologically relevant odorant detected, and the portion of the OB that processes this information (Hansen et al. 2003). The evidence indicates that amino acid odorant information in the channel catfish is transduced both by ciliated ORNs through the c-AMP second-messenger pathway and microvillous ORNs through the IP₃ pathway. Other recent reports confirm these findings for amino acid odorant transduction in other aquatic organisms (Delay and Dionne 2002; Manzini et al. 2002; Sato and Suzuki 2001).

The logic for the selection in the present experiments of the individual amino acids used in the search for amino acid-responsive neurons (the L-isomers of sodium glutamate, arginine hydrochloride, alanine, and methionine) was based on the results of previous biochemical binding (Bruch and Rulli

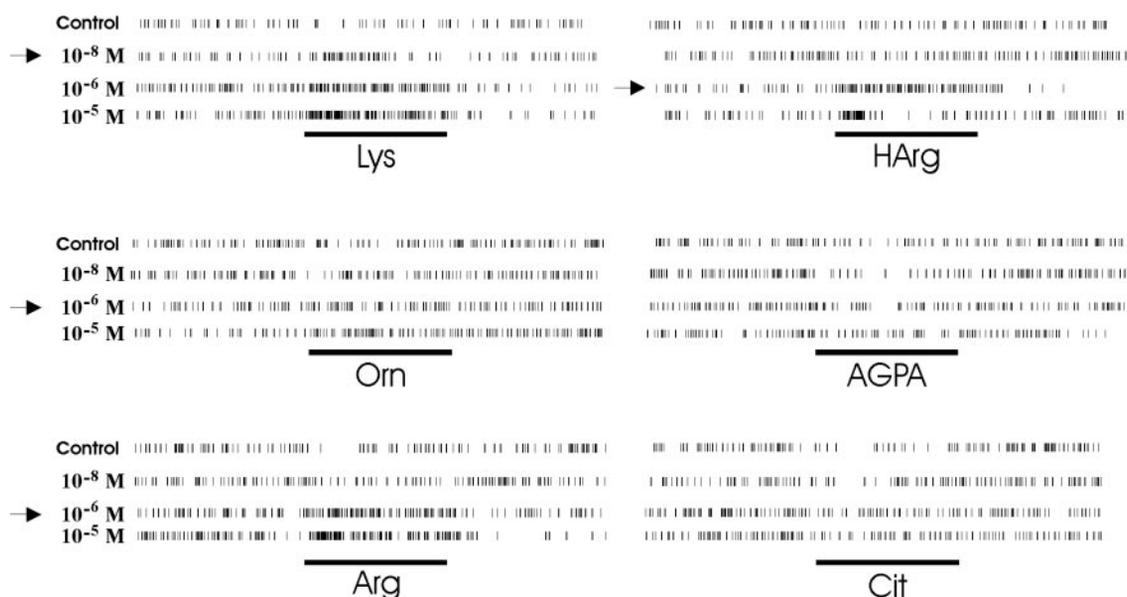


FIG. 5. Responses of a representative OB unit (cell 1 in Fig. 6) to basic amino acids. Odorant concentrations are listed adjacent to each record; arrow indicates the lowest odorant concentrations that resulted in an excitatory response for that unit. Neither AGPA nor Cit excited the unit. Odorant onset and duration (3 s) are indicated by the line below each control trace.

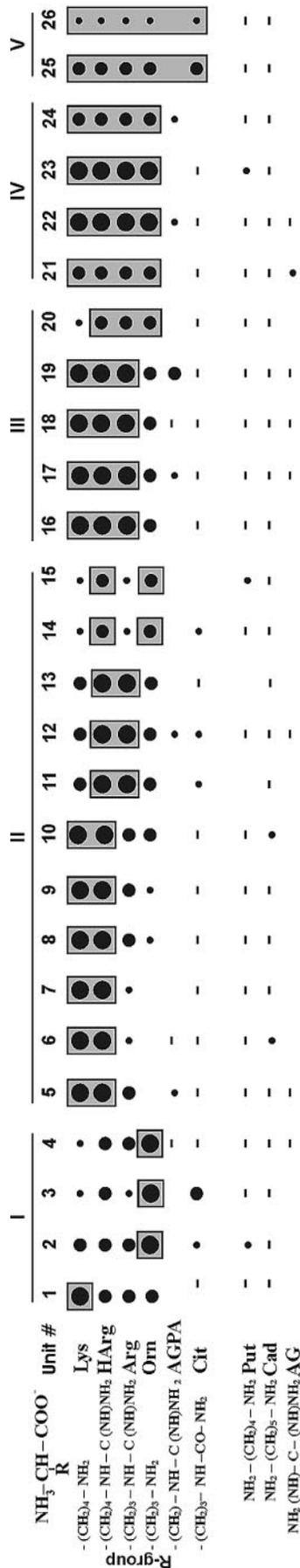


FIG. 6. Electrophysiologically derived excitatory thresholds of 26 OB Group I neurons to basic amino acids and derivatives. Roman numerals indicate the number of basic amino acid odorants that resulted in an excitatory response at the lowest concentration for each unit. Dots within the shaded boxes indicate both the concentrations and specific odorant(s) that resulted in an excitatory response at the lowest test concentration for each OB unit. - indicates those neurons that were not excited by the particular odorant tested at 10^{-4} M.

1988), electrophysiological cross-adaptation (Caprio and Byrd Jr 1984) and electrophysiological responses to amino acid mixtures (Caprio et al. 1989; Kang and Caprio 1991) performed in this species. The results of all 3 types of prior experiments collectively indicated relatively independent molecular olfactory receptors for acidic, basic, and neutral amino acids in the olfactory organ of the channel catfish. The data also suggested that relatively independent olfactory receptors also exist for neutral amino acids with short (SCN) and long (LCN) side chains, respectively. Biochemical (Brown and Hara 1981; Cagan and Zeiger 1978; Lo et al. 1991; Rehnberg and Schreck 1986; Rhein and Cagan 1983), electrophysiological cross-adaptation (Michel and Derbidge 1997), and calcium-imaging (Friedrich and Korsching 1997; Fuss and Korsching 2001) studies of ORNs in other teleostean species have provided confirming evidence for these broad categories of olfactory receptor types in teleosts. The present results also indicate relatively independent olfactory receptor sites for ILCNs and bCNs.

Response time consideration

Mitral cell responses to amino acids in zebrafish were recently described as being nonstationary, in that selectivity of individual mitral cells changed over 2.2 s of the response, which resulted in a declustering of the response types observed during early portions (initial 500 ms, approximately) of the response (Friedrich and Laurent 2001; Laurent et al. 2001). We therefore specifically addressed this question by analyzing the response of Group I bulbar neurons in the channel catfish to amino acids during the first and third seconds of the 3-s response to determine whether significant changes in unit selectivity occurred. The results clearly indicated that the response selectivity of the vast majority of OB units in catfish was maintained between the first and third seconds of the response.

Although species differences may exist, it is likely that zebrafish and channel catfish process amino acids similarly within the OB. Ongoing behavioral studies indicate that zebrafish (T. Valentincic, personal communication) have abilities similar to those of catfish (Valentincic et al. 1994, 2000) to discriminate amino acids by olfaction. The reason for the apparent discrepancy between the previous report in zebrafish and the present study in the channel catfish is currently unknown.

The selectivity of Group I OB units to amino acids

One portion of the electrophysiological results presented here, those for the Group I units, which constituted 37% of the tested OB units, showed an excitatory response range similar to the results of the amino acid binding studies (listed above), indicating clear differences in single-cell specificities for different classes of L- α -amino acids across different OB neurons recorded in vivo. These Group I units were highly selective for the type of amino acid [i.e., possessing either an acidic (A), basic (B), neutral with short side chain (SCN), or neutral with a long side chain (LCN)]. All 91 of these units were excited from 10^{-6} to 10^{-4} M by only one of the 4 amino acid odorants representing 4 different categories of amino acid side chains. Further, the 154 Group II units recorded showed varying

specificities to the 4 types of amino acids from being rather specific, responding to a second type of amino acid only at a 100-fold higher concentration (i.e., Arg and Glu Group II units), to responding to a second type of amino acid at one log unit higher concentration than the most stimulatory amino acid to that unit (Met Group II units).

Although the excitatory responses of single OB neurons were studied in the present report, suppressive responses (i.e., significant decline in the number of action potentials from ongoing spontaneous activity) were often evident. Suppression is likely a mechanism for contrast enhancement (Schild and Restrepo 1998) and not directly related to the coding of olfactory information that is processed at the next higher synaptic level (Duchamp-Viret and Duchamp 1997). For example, Fig. 1 shows the responses of an OB unit described as an "Arg unit" that is excited by Arg (a basic amino acid), but is suppressed by neutral (Met and Ala) and acidic (Glu) amino acids; further the "Glu unit" that is excited by Glu (an acidic amino acid) is suppressed by both Ala (a neutral amino acid) and Arg (a basic amino acid). It is the synchronized excitatory response that is most efficient in effectively driving postsynaptic neurons within the FB (Nikonov et al. 2002). Further, it was the excitatory response of OB units that was odor specific and was the basis of the previously described odotopic map (Nikonov and Caprio 2001).

The following discussion relates to the excitatory molecular response range (EMRR) (Mori and Yoshihara 1995) of the 69 Group I units that were tested with an expanded list of amino acid and related odorants to obtain a more precise examination of excitatory spectrum. The EMRR of single mitral cells within the OB reflects both the tuning specificities of the ORNs that innervate a particular mitral cell and the lateral interactions occurring between mitral cells and local interneurons, such as granule cells (Yokoi et al. 1995). Further, how the EMRRs of OB neurons within the OB relate to the results of behavioral conditioning experiments to amino acids are indicated.

LINEAR VERSUS BRANCHED NEUTRAL AMINO ACID UNITS. The present results based on electrophysiological thresholds using an expanded list of neutral amino acids indicated that a subset of bulbar units distinguished ILCNs from bCNs (Fig. 3A vs. B). With increasing amino acid concentration, responses broadened such that these single OB neurons responded selectively to neutral amino acids having either linear or branched side chains, but not to amino acids with acidic or basic side chains. Prior studies in the channel catfish provided evidence for the existence of olfactory receptor sites for LCNs being different from those for SCNs (Bruch and Rulli 1988; Caprio and Byrd Jr 1984); however, electrophysiological evidence for independent olfactory receptor sites for bCNs was lacking. The present electrophysiological results of a broadening of OB unit selectivity to neutral amino acids with increasing stimulus concentrations reflect the related finding of a recruitment of additional responsive glomeruli with increasing odor intensities (Friedrich and Korsching 1997; Joerges et al. 1997; Meister and Bonhoeffer 2001; Rubin and Katz 1999). Further, assuming that catfish (Caprio 1978) and zebrafish (Michel and Lubomudrov 1995) have similar olfactory sensitivities to amino acids, the present results indicate that the electrophysiological recording of OB unit activity is a more sensitive technique than Ca^{2+} imaging (Fuss and Korsching 2001) in detecting odorant-

induced responses. A 10- μ M concentration was required for the discrimination of amino acids in the Ca^{2+} -imaging experiments in zebrafish, whereas 0.01 μ M was sufficient for discrimination of different amino acids in the present single-cell electrophysiological experiments (Figs. 3, 4, and 6).

More recently, a study of olfactory discrimination of amino acids in bullhead catfish clearly indicated significant differences between responses to bCNs versus ILCNs (Valenticinc 2000), reflecting likely differences in olfactory receptors and bulbar processing for these different types of amino acids. Although differences were clearly evident in electrophysiological thresholds between ILCNs and bCNs in different groups of OB neurons in the present study, the majority of these OB neurons were responsive to both types of amino acids, albeit at different stimulus concentrations. This finding is similar to that obtained in a previous study of mitral cell responses in the rabbit to a homologous series of normal and iso-fatty acids (Imamura et al. 1992). In both catfish and rabbit, OB neurons were observed that were highly responsive to compounds consisting of a 4–5 carbon skeleton that were either linear or branched. OB neurons in catfish and rabbit that responded excitedly to branched amino acid and fatty acid molecules, respectively, were often also activated by the linear form of the molecule.

SCN UNITS. The 21 Ala units (i.e., those with lowest threshold to Ala) responded least specifically and were excited by 2 other types of amino acids. However, the vast majority (138/154) of the Group II units were selective to a specific amino acid type at 10^{-6} M and likely below. These electrophysiological results are paralleled by the ability of catfish to discriminate behaviorally among these categories of amino acids. For example, catfish conditioned to L-Ala (an SCN) discriminated acidic, basic, and several LCNs from L-Ala, but did not readily discriminate other SCNs from Ala (Valenticinc 2000). These results are similar to those obtained in the zebrafish using Ca^{2+} -imaging techniques, in that some glomeruli were observed in the zebrafish olfactory bulb that preferred neutral amino acids with short side chains (Fuss and Korsching 2001).

The threshold for Ala was lowest or equal to the lowest threshold for the expanded list of SCN amino acids tested for all 12 OB units that were initially solely excited by Ala (SCN) [and not by Met (LCN), Arg (B), or Glu (A) (Fig. 4)]. Eleven of these 12 OB units in channel catfish were also excited by L-serine (Ser), and 5 shared the same electrophysiological threshold for Ala and Ser. Ser was reported previously to also have a high degree of response similarity to Ala for OB units in both rainbow trout (MacLeod 1976) and goldfish (Meredith 1981). Both biochemical binding (Brown and Hara 1981; Bruch and Rulli 1988; Cagan and Zeiger 1978; Lo et al. 1991; Rehnberg and Schreck 1986; Rhein and Cagan 1983) and electrophysiological cross-adaptation (Caprio and Byrd Jr 1984) experiments in a variety of teleosts suggested that Ala and Ser bind to some common molecular olfactory receptors, and that these receptors were distinct from those that bind other types of amino acids. The possibility that choosing a different SCN search stimulus than Ala might have resulted in the finding of OB units with lowest threshold to other SCNs cannot be ruled out; however, selecting Met as the search stimulus for LCNs did not eliminate finding units with higher sensitivity to bCNs (Fig. 3), nor did choosing Arg as the search stimulus for

basic amino acids eliminate the finding of OB units that were more sensitive to Lys (Fig. 6).

B UNITS. The basic amino acid units, those with lowest electrophysiological thresholds to Arg and Lys, the major basic amino acids in nature, were also distinct in that neither A, SCN, nor LCN amino acids were as effective in releasing swimming activity as was Arg or Lys in Arg- conditioned and Lys-conditioned catfish, respectively (Valenticic et al. 1994, 2000). These results are consistent with previous amino acid receptor binding (Bruch and Rulli 1988; Cagan and Zeiger 1978; Lo et al. 1991; Rehnberg and Schreck 1986), cross-adaptation (Caprio and Byrd Jr 1984), mixture (Caprio et al. 1989; Kang and Caprio 1991), and calcium-imaging (Friedrich and Korsching 1997) studies, indicating the independence of the basic from other types of amino acid receptors in fish olfaction. Further, the existence of independent receptor sites and neural pathways for Arg and for Lys were indicated in behavioral experiments in the channel catfish where these basic amino acids were distinguishable (Valenticic et al. 1994). The present results suggest that a requirement in the channel catfish for activation of ORNs expressing basic amino acid receptor(s) at relatively low ($\leq \mu\text{M}$) concentration is that the odorant molecule be an α -amino acid possessing a side chain containing 3–4 methylene groups with a terminal amide or guanidinium group (e.g., Lys, HArg, Arg, and Orn, but not AGPA or Cit, which lack these; Fig. 6). Because the guanidinium group itself is planar, the covalent bond connecting it to the terminal nitrogen in the molecule can likely bend and circumvent steric hindrance at the binding site. Fewer than 3 methylene groups in the side chain before the terminal amide or guanidinium group is generally insufficient for activation (e.g., AGPA); further a carbonyl oxygen substituted in the guanidinium moiety generally eliminates the response (e.g., Cit; Fig. 6). Citrulline was also less effective than Lys in displacing [^3H]-L-Arg binding to the olfactory basic amino acid receptor in the goldfish (Specia et al. 1999).

OB unit selectivity and behavior to amino acids

A recent electrophysiological study of the zebrafish olfactory bulb reported that mitral cell responses to amino acids were not highly specific (Friedrich and Laurent 2001; Laurent et al. 2001). However, the present experiments identified in the channel catfish OB units (Group I) that were highly selective for amino acid type (acidic, basic, neutral). A similar grouping of amino acids was indicated in competitive binding assays with olfactory cilia in salmonids (Rhein and Cagan 1983) and cross-adaptation experiments of olfactory receptor responses in catfish (Caprio and Byrd Jr 1984). Further, in the present experiments in catfish, different populations of Group I units that were highly selective for neutral amino acids were also selective for type of neutral amino acid (i.e., those with short and long side chains, respectively); these latter units even showed specificity for type of long side chain, either linear or branched. Even the Group II units that responded to more than one type of amino acid exhibited selectivity in that specific units of a particular type were not responsive to particular other types of amino acids. It is rather intriguing that the present electrophysiological results of amino acid specificities of single OB units in the channel catfish are highly similar to those

reported from calcium-imaging studies in the OB of zebrafish (Friedrich and Korsching 1997). Further, recent electrophysiological data obtained in zebrafish also provide evidence for OB unit selectivity to type of amino acid over the first 0.5 s of the response before a declustering of odor representations (Friedrich and Laurent 2001).

An advantage of the present electrophysiological testing of the specificity of OB units to amino acids in the channel catfish is that evidence exists for the learned behavioral discrimination of many of the same amino acids in the same (Valenticic et al. 1994) and in a closely related (Valenticic et al. 2000; recently reidentified as the black bullhead, *Ictalurus melas*; T. Valenticic, personal communication) species of catfish. In these previous behavioral studies, the concentrations of amino acids that contacted the fish were estimated in the range of 10^{-5} to $\leq 10^{-7}$ M. The present electrophysiological results can thus be directly compared with the behavioral data to assess whether the OB unit specificities determined herein are sufficient to account for the behavioral results. Both species of catfishes were capable of discriminating behaviorally the majority of the amino acids tested. The presently described OB unit specificities appeared sufficient to provide the basis for majority of this behavioral discrimination. Both channel and bullhead catfishes quite reliably discriminated behaviorally acidic, basic, and neutral amino acids from each other as did the Group I OB units determined electrophysiologicaly (this report) in the channel catfish; further, even the Group II units in the channel catfish showed sufficient discrimination across the different types of amino acids to participate in the behavioral discrimination. The presently described OB unit specificities also likely account for the ability of catfish to discriminate behaviorally compounds included within specific types of amino acids (e.g., channel catfish: Lys from Orn, Arg from AGPA; Fig. 6). Similarly, for those amino acids that catfish did not discriminate behaviorally (e.g., nLeu, nVal, and Met), there was little evidence for differences in odorant specificity of OB units for these compounds (Figs. 3 and 6).

Temporal patterning of OB unit responses, which was not analyzed in the present investigation, may contribute further to the discrimination of amino acids (Friedrich and Laurent 2001), especially for those compounds whose OB unit specificities could not account for the behavioral discrimination [e.g., Arg and Lys in the channel catfish (Valenticic et al. 1994)]; however, the more recent study in bullhead catfish indicated that in some tests Arg and Lys could not be discriminated (Valenticic et al. 2000). The present investigation therefore indicates that the specificities of the OB units in the channel catfish provide sufficient detail concerning the side-chain structure of many different L- α -amino acid odorants to allow for their behavioral discrimination.

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