

Olfactory Discrimination of Amino Acids in Brown Bullhead Catfish

Tine Valentinčič, Jutra Metelko, Damijana Ota, Vlado Pirc and Andrej Blejec¹

Department of Biology, University of Ljubljana and ¹National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

Correspondence to be sent to: Tine Valentinčič, Department of Biology, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia.
e-mail: tine.valenticic@uni-lj.si

Abstract

Olfactory discrimination of amino acids was investigated in brown bullhead catfish (*Ameiurus nebulosus*). Based on the magnitude of the observed food search activity of catfish conditioned to single amino acids, the tested compounds were classified as being detected by the catfish as equal to, similar to, or different from the conditioned stimulus. L-Proline (L-Pro)-conditioned brown bullhead catfish discriminated all amino acids from L-Pro, but catfish conditioned to L-valine (L-Val) and L-isoleucine (L-Ile) did not discriminate L-Val from L-Ile nor L-Ile from L-Val; however, all other amino acids tested were always discriminated from these two compounds. Catfish conditioned to L-alanine (L-Ala) discriminated basic, acidic and several neutral amino acids with long side-chains (LCNs) from L-Ala; however, they did not always discriminate L-Ala from all neutral amino acids with short side-chains (SCNs). The L-norleucine (L-nLeu)-conditioned fish responded to L-norvaline (L-nVal), L-methionine (L-Met) and L-Ala similarly to L-nLeu, indicating that these amino acids are detected as similar or identical to L-nLeu. L-nLeu was, however, discriminated from L-Ala in L-Ala-conditioned catfish. Interestingly, L-leucine (L-Leu) was discriminated from the conditioned stimuli, L-Ala, L-Ile and L-Val, indicating independent receptors for L-Leu. Although conditioned catfish discriminated other amino acids from L-arginine hydrochloride (L-Arg), in some tests they were unable to discriminate L-Arg from L-lysine hydrochloride (L-Lys). These results imply the existence of independent olfactory receptive pathways for: (i) L-Pro; (ii) basic amino acids (L-Arg and L-Lys); (iii) L-Leu; (iv) other neutral amino acids with branched side-chains (L-Ile and L-Val); (v) neutral amino acids with long linear side-chains (L-nLeu, L-nVal and L-Met); (vi) neutral amino acids with short side-chains; and (vii) amino acids with sulfhydryl groups (L-Cys and L-homoCys).

Introduction

Fish detect amino acids by their taste and olfactory systems which, based on extracellular electrophysiological recordings, exhibit generally different chemical specificities (Caprio, 1978; Marui and Caprio, 1992; Hara, 1992). The olfactory system is broadly tuned, releases feeding excitation and enables animals to learn and identify odors, such as the formation of a chemical search image (Atema *et al.*, 1980; Little, 1981; Valentinčič *et al.*, 1994). The taste system, in contrast, is more narrowly tuned and provides direct input to the brainstem to release the reflex behaviors of turning and biting/snapping, and in catfish, also releases feeding excitation (Finger, 1978; Morita and Finger, 1985; Marui and Caprio, 1992; Valentinčič and Caprio, 1994b). The taste system in fish is not involved in conditioning to chemical stimuli and it has not been shown to provide sensory inputs allowing discrimination of chemical stimuli in laboratory assays used to date (Valentinčič and Caprio, 1994b). Heart-rate conditioning techniques provided the first experimental evidence for olfactory discrimination of non-conditioned from conditioned amino acids, as channel catfish were unable to discriminate different odors when the nose was plugged (Little, 1981). The first reported evidence

of olfactory discrimination based on conditioning in free-swimming fish was in brown bullhead catfish (*Ameiurus nebulosus*), which learned to discriminate glutamine from other amino acids (Atema, 1977).

Receptor binding (Cagan and Ziegler, 1978; Rehnberg and Schreck, 1986; Kalinoski *et al.*, 1987; Bruch and Rulli, 1988), electrophysiological cross-adaptation (Caprio and Byrd, 1984; Ohno *et al.*, 1984; Sveinsson and Hara, 1990) and mixture (Caprio *et al.*, 1989; Kang and Caprio, 1991) studies indicated independent olfactory receptor sites for acidic, basic, imino and neutral amino acids in fish. Channel catfish are capable of discriminating acidic, basic, imino and neutral amino acids (i.e. 'across-class' discrimination) from each other (Valentinčič *et al.*, 1994). This previous study also indicated that amino acids within the category of basic amino acids could be discriminated from other members of the same class of amino acids; the question remained, however, as to whether the olfactory system of catfish can discriminate specific compounds comprising the large neutral amino acid group that could be divided into different categories: SCN, short chain neutral; LCN-linear, long chain neutral-straight chain; and LCN-branched, long

chain neutral-branched amino acids. A previous electrophysiological study of the responses of olfactory receptor neurons of the channel catfish to complex mixtures of amino acids indicated the existence of different receptors for neutral amino acids with short and long side-chains, respectively (Kang and Caprio, 1991). The present investigation was designed to determine similarities and differences between the detection of amino acids by the olfactory systems of the channel (Valentinčič and Caprio, 1994a; Valentinčič *et al.*, 1994) and bullhead catfishes, different species within the family Ictaluridae. We examined whether the olfactory system of ictalurid catfish is capable of providing sufficient information to allow for the discrimination of specific amino acids from others within the neutral amino acid, basic amino acid and imino acid stimulus classes.

Materials and methods

Animals

Ameiurus nebulosus, either raised in ponds near Ljubljana (Slovenia) or purchased from a fish farm (Crna Mlaka near Karlovac in Croatia), were transported to the aquarium room located on the ground floor of the University of Ljubljana Biology building located 200 m from the nearest road. Distance from sources of low frequency vibrations (e.g. traffic) is essential to avoid escape behavior of the catfish (T. Valentinčič, unpublished data). The catfish were treated for ich (*Ichthyophthyrus multifilis*) with malachite green and maintained in storage tanks for periods of at least 6 months prior to experimentation. To provide a sufficient time period for the catfish to acclimate to the aquarium environment, they were transferred from storage tanks to aquaria 2 months prior to the initiation of the conditioning procedures. Fifteen 80 l aquaria, each containing one catfish, were used for each experiment.

Stimuli

The following amino acids and their analogs and isomers were tested in this study.

1. Neutral amino acids with short side-chains: glycine (Gly); L-alanine (Ala); L-serine (Ser); L-threonine (Thr); and β -alanine (β -Ala).
2. Glycine derivatives: sarcosine (Sarc) and betaine (Bet).
3. Amino acids with short side-chains containing a sulfhydryl group: L-cysteine (Cys); L-homocysteine (homoCys); and Cys derivatives.
4. Neutral amino acids with long side-chains: L-valine (Val); L-isoleucine (Ile); L-norvaline (nVal); L-norleucine (nLeu); L-leucine (Leu); and L-methionine (Met).
5. D-amino acids: D-Ala; D-Cys; D-Val; D-Leu; and D-nVal.
6. Basic amino acids: L-arginine (Arg) and L-lysine (Lys).
7. Acidic amino acid: L-aspartic acid (Asp).
8. Imino acid: L-proline (Pro).

Stimulus delivery and dilution

Chemical stimuli were added using air pressure to a removable Pasteur pipette attached to a syringe above each aquarium. The syringe was connected through Tygon tubing (0.8 mm inner diameter) to a second 5 ml syringe 2.5–4 m away from the aquaria. To minimize possible sources of contamination, the pipettes were washed with hot water five times for 1 min each and equilibrated in dechlorinated tap water five times for 2 min each prior to each test. Solutions (10 mM) of the finest grade amino acids available [Sigma Chemical Corp. (St Louis, MO) and Fluka Chemica-BioChemica (Buchs, Switzerland)] were prepared <40 min before each experiment. L-Val (Sigma, No. V-0500 Lot No. 77H0511) and L-Ile (Fluka, No. 58879, analysis No. 270336 889) were analyzed for impurities using a 421A amino acid analyzer (PE Applied Biosystems, Foster City, CA) and reference chemicals (sequential grade) from the same source. L-Val was determined to be 98.96% L-Val, 0.35% L-Ile and 0.68% L-Thr; L-Ile was determined to be 99.70% L-Ile, 0.28% L-Val and 0.02% L-Th. There were no other amino acid contaminants detected in the two previous samples. Solutions of amino acids were added to the surface of each aquarium into the region of the turbulent water surface caused by the aeration system in order to mask the slight mechanical cue associated with stimulus addition. Video-recordings of dispersed clouds of milk over time following addition of an aliquot to an aquarium permitted the adjustment of the aquaria airflows to create the currents to carry the test stimulus into the fish shelters in <30 s. During the experiments, the test solution was diluted to yield a maximum peak concentration of $0.3\text{--}3 \times 10^{-5}$ M in the center of the stimulus eddies (i.e. odor plumes) near the fish 5–25 s after stimulus injection (Moore *et al.*, 1989; Valentinčič and Caprio, 1994a). The final concentration of the test stimuli <2 min after the injection was estimated to be $\sim 10^{-7}$ M. Catfish that swim through a stimulus eddy always encounter gradients, i.e. rapidly increasing concentrations of chemicals towards and decreasing concentrations away from the center of the eddy. Provided that the stimulus concentrations in the center of the stimulus eddy are above behavioral threshold, feeding behavior ensued. Neither the duration nor the intensity of the feeding behavior increases with further increase of the stimulus concentration within the eddy. It is the number of stimulus eddies encountered that potentially influences the intensity and duration of the feeding behavior (Valentinčič and Caprio, 1994b).

Conditioning experiments

For experiments in aquaria without shelters, a food reward was presented 90 s after the addition of the conditioned stimulus, a specific amino acid solution; for conditioning experiments with catfish in aquaria with shelters (black plastic tubes 20 cm long, 9 cm in diameter), a food reward

was presented 120 s after addition of the conditioned stimulus. The additional 30 s were required for the stimulus eddies to reach inside the shelters containing the fish. A maximum of four conditioning sessions was carried out per day. The ability of brown bullhead catfish to associate the odor of a specific (i.e. 'conditioned') amino acid with a food reward which caused the fish to leave its shelter and search for food enabled the classification of test amino acids as identical, similar to, or different in quality from the conditioned stimulus. Whenever the number of turns released by the test stimulus was not significantly different from that evoked by the conditioned stimulus, the substance was considered to be detected as equal to the conditioned stimulus. In the paradigm 2 experiments, the test stimuli that were totally ineffective were detected as different from the conditioned stimulus, and the test stimuli that were sometimes effective and other times ineffective at releasing food-searching behavior were considered to be detected as similar to the conditioned stimulus.

Since both taste and olfactory systems of fish detect amino acids, it was necessary to perform a subset of experiments in anosmic catfish to determine if the taste system alone is sufficient to allow for amino acid discrimination. The olfactory organs were bilaterally extirpated under anesthesia during continuous gill irrigation with 1:8000 solution of 3-aminobenzoic acid ethyl ester methane-sulfonate. The animals were tested 1–3 months after the surgery. At the termination of the behavioral experiments, the fish were inspected for regeneration of the olfactory lamellae. Reported are results of chemosensory experiments performed in brown bullhead catfish, which remained anosmic and showed no regeneration of olfactory lamellae and those from brown bullhead catfish with regenerated olfactory lamellae.

Data analyses

The number of $>90^\circ$ turns of each fish during the initial 90 (or 120) s following the addition of either a water control or a test stimulus was video-recorded (JVC, sVHS, GFS550) and quantified after the experiment. The number of turning responses after stimulation with the conditioned stimulus was compared with that after stimulation with individual test (i.e. unconditioned) amino acids. The data obtained in the first series of tests with different amino acids were sorted from the largest to the smallest response (number of turns) (Figure 2). To exclude the possibility that changes in saliency of the conditioned stimulus and stimuli similar to the conditioned stimulus influenced the results, test series with different amino acids were replicated two or three times. The same sorting order from the first test series was maintained for presentation of the second and third test series. The Wilcoxon sum of ranks test was used to identify whether the behavioral responses to the non-conditioned amino acids were significantly different from the responses to the conditioned stimulus.

Results

Responses of naive and conditioned brown bullhead catfish to single amino acids

Brown bullhead catfish have small eyes and, in contrast to channel catfish, do not respond to visual stimuli with either feeding or escape behavior. The most potent stimuli that release escape behavior in brown bullhead catfish are low frequency vibrations. When vibrations were avoided, brown bullhead catfish responded to feeding stimuli with feeding behavior. Naive brown bullhead catfish, which hid in shelters and were not conditioned to react to specific amino acid stimuli, responded little to stimulation with single amino acids. The behavioral response (which lasted <1 min) of unconditioned brown bullhead catfish to single tested amino acids consisted of barbel movement, orienting posture and motor unrest while remaining inside their shelters. The few times naive brown bullhead catfish responded to stimulation by an amino acid, the number of $>90^\circ$ turns was <10 , and the median number of turns was zero. Brown bullhead catfish conditioned to single amino acids responded to food stimuli with barbel movements and twitches, exploratory search with barbels at the entrance of the shelter, orienting body-upwards posture outside the shelter and rapid swimming. During the conditioned food search, unlike the response to non-conditioned stimuli, the fins of the fish remained erect as it swam rapidly making abrupt turns.

In the conditioning experiments, <30 s was necessary for stimulus eddies (plumes) to reach the fish. During the search swim, the fish encountered the stimulus eddies for an additional 30–60 s. Each time the eddy was encountered, the fish either snapped or turned and accelerated the swim. During repeated stimulation with a single amino acid followed by a food reward at the end of the test, 3-year-old brown bullhead catfish increased swimming activity during 30–50 conditioning trials, from medians of 0–40 to up to 60 turns (L-Ala conditioning; Figure 1A). Six-year-old brown bullhead catfish increased swimming activity in a similar number of conditioning trials to 20–30 turns (L-Arg HCl conditioning, Figure 1B). Conditioning stimuli released the complete sequence of appetitive events. After five to ten conditioning trials, the magnitude of the conditioned response was significantly greater than that of naive animals. When conditioning was interrupted just prior to the 30th and 50th sessions, the response to L-Ala remained high (Figure 1A); cessation of the conditioning procedures for 1–3 months subsequent to 30–50 conditioning trials did not significantly reduce the response to the conditioned stimulus when the trials resumed.

Conditioned responsiveness of brown bullhead catfish

In aquaria without shelters

In the experimental paradigm where the animals were

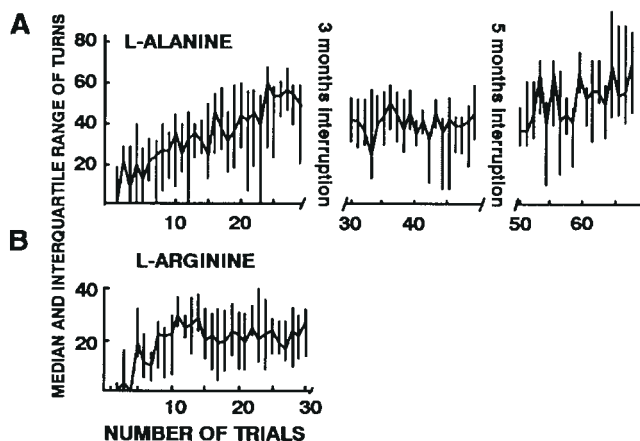


Figure 1 Swimming behavior of brown bullhead catfish during conditioning to the amino acids L-alanine (A) and L-arginine HCl (B) measured as the number of $>90^\circ$ turns of the fish within 90 s of stimulus presentation. Vertical bars indicate the median and interquartile range of turns. The effects of interruptions upon the conditioning procedures for three to five months are shown in (A).

maintained and tested without shelters available in the aquaria, the number of turns of brown bullhead catfish following an amino acid presentation was always greater than zero; catfish without shelters swam after every chemical stimulation which was above behavioral threshold. A median response to the conditioned stimulus was usually more than twice as large as the response to the non-conditioned test amino acid stimulus (Table 1A, B, lines A, C, D; Figure 2). The number of turns after repeated stimulations with the same non-conditioned stimuli that were detected as different from the conditioned stimulus differs randomly; Spearman correlation coefficients indicate no correlation between trials (Figure 2, trials 1–3).

In the paradigm without shelters, the turning response to L-Pro, an imino acid, was two to three times greater than the response after stimulation with the non-conditioned α -amino acid stimuli and the water control (Table 1A, B, line A). There was no statistical difference between the response to the water control and to the non-conditioned amino acids. Catfish conditioned to L-Ile, a neutral amino acid with a branched side-chain, always responded equally well to L-Val, a similarly structured amino acid; however, the number of turns after stimulation with all other amino acids in the first, second and third test series were significantly smaller (Figure 2; Table 1A, B, line C). Similarly, catfish conditioned to L-Val responded equally to L-Ile and significantly less to other amino acids (Table 1A, B, line D).

In aquaria provided with shelters

Catfish remained in shelters until stimulated by either the conditioned stimulus or stimuli detected as equal or similar to the conditioned stimulus (Table 1A, B, lines B, E–G). Catfish conditioned to L-Ala, a neutral amino acid with a short side-chain, occasionally responded to other similarly structured amino acids, including Gly, L-Ser, L-Cys and

β -Ala. Initially, during the first test series, these responses were not significantly different from the response to the conditioned stimulus; however, in subsequent tests, catfish discriminated these stimuli from the conditioned stimulus (Table 1A, B, line F). On some occasions in L-Ala-conditioned fish, neutral amino acids with larger side-chains, such as L-nLeu, L-Met and L-nVal, also released short bursts of swimming activity with a median number of turns <10 . L-Ala conditioned catfish did not, however, leave their shelters after stimulation with neutral amino acids containing branched side-chains, or with L-Pro, L-Arg HCl or L-Thr. The response to L-nLeu in L-Ala-conditioned catfish was significantly smaller than the conditioned response to L-Ala; however, catfish conditioned to L-nLeu responded equally well to L-Ala, L-nVal and L-Met, but poorly to the other test amino acids (Table 1A, B, line E). L-Leu tested in catfish conditioned to L-Ile, L-Val, L-Ala and L-nLeu always released significantly less swimming activity than the conditioned stimuli (Table 1A, B, lines C–F).

L-Cys-conditioned catfish were also highly responsive to DL-homoCys, an amino acid with an extra methylene group compared to L-Cys (Table 1A, B, line G). The number of turns in response to DL-homoCys stimulation was not significantly different than during L-Cys stimulation. Although these fish responded initially to several other analogs of L-Cys, such as D-Cys, *N*-acetyl-L-Cys, *S*-methyl-L-Cys and L-Ala, a repeat of the tests indicated that compounds other than DL-homocysteine were significantly less stimulatory than L-Cys, the conditioned stimulus. Neutral amino acids possessing branched side-chains (e.g. L-Ile), basic amino acids and the imino acid L-Pro did not stimulate L-Cys-conditioned brown bullhead catfish to leave their shelters.

The basic amino acid, L-Lys HCl, stimulated L-Arg HCl-conditioned catfish to exit shelters and to swim and turn, but the magnitude of the response to the non-conditioned amino acid, L-Lys HCl, was significantly less than that to the conditioned stimulus, L-Arg HCl (Table 1A, B, line B).

Olfactory discrimination: anosmic catfish versus catfish with regenerated olfactory lamellae

The ability to discriminate chemical stimuli was tested in 13 brown bullhead catfish 3 months after bilateral surgical removal of their olfactory organs. Three of these catfish which remained anosmic (i.e. the surgical openings were overgrown by connective tissue and skin) were unable to discriminate L-Arg HCl from the conditioned L-Ala stimulus (Figure 3A). Of the remaining ten fish, the entire olfactory rosette regenerated in four fish, several olfactory lamellae were regenerated in four other fish and a single olfactory lamella regenerated in the two remaining fish. All ten of these catfish conditioned to the neutral amino acid L-Ala were able to discriminate the basic amino acid L-Arg HCl from L-Ala (Figure 3B).

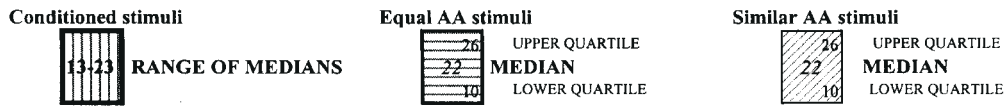
Table 1 Swimming (turning) responses of amino acid-conditioned brown bullhead catfish to different amino acids

A FIRST TEST

		TEST STIMULI																			
		IMINO			BASIC			BRANCHED			LONG CHAIN			SHORT CHAIN			SULHYDRYL			OTHER	
		L-Pro	L-Arg HCl	L-Lys HCl	L-Ile	L-Val	L-Leu	L-nLeu	L-nVal	L-Met	L-Ala	Gly	L-Ser	beta-Ala	L-Cys	DL-homo Cys	D-Cys	D-Ala	L-Thr		
CONDITIONING STIMULI	A) L-Pro N=15	31-50			11 9 *	26 21 *	14 7 *	15 11 *	20 10 *		14 12 *	12 14 *			16 13 *				19 13 *		
	B) L-Arg HCl N=11		22-42	48 9 *	7 0 *				12 0 *		11 0 *	31 0 *	13 0 *		4 0 *	5 0 *			0 0 *		
	C) L-Ile N=11	41 9 *	14 8 *		16-46 31	48 37	33 12 *	33 10 *	22 10 *	31 15 *	34 12 *	12 11 *			18 10 *			22 8 *			
	D) L-Val N=11	3 1 *	5 2 *		16 14	13-23 13	22 4 *	10 3 *	14 8 *	17 3 *	3 1 *	12 3 *			13 3 *			5 4 *			
	E) L-nLeu N=11	0 0 *	3.5 0 *	0 0 *	13 1 *	13 3 *	6 1 *	18-29 13 *	33 12 *	20 12 *	33 23	22 9 *	14 0 *	23 10 *	18 2 *			7 0 *	8 1 *		
	F) L-Ala N=9	0 0 *	21 0 *		22.5 0 *		13 3 *	14 *	0 *	37.5 6 *	21.5 2.5	39-66 47	45.3 37	58 24 *	39.5 35	34.5 24 *		39.5 4 *	0 0 *		
	G) L-Cys N=8		0 0 *	11.5 0 *	0 0 *					17 5 *	26 22 *				26-28 27	26 14.3	12.5 3 *				

B SECOND TEST

		TEST STIMULI																			
		IMINO			BASIC			BRANCHED			LONG CHAIN			SHORT CHAIN			SULHYDRYL			OTHER	
		L-Pro	L-Arg HCl	L-Lys HCl	L-Ile	L-Val	L-Leu	L-nLeu	L-nVal	L-Met	L-Ala	Gly	L-Ser	beta-Ala	L-Cys	DL-homo Cys	D-Cys	D-Ala	L-Thr		
CONDITIONING STIMULI	A) L-Pro n=15	21-50			23 13 *	22 12 *	15 12 *	12 10 *		19 14 *	12 10 *			15 12 *				15 13 *			
	B) L-Arg HCl n=11		20-42	34 12	8 5 *			9 0 *		15 0 *	24 0 *	15 0 *		8 0 *	9 0 *			4 0 *			
	C) L-Ile n=11	42 10 *	14 8 *		16-46 25	33 11 *	30 18 *	16 17 *	23 6 *	18 9 *	37 15 *	23 5 *		12 5 *			14 8 *				
	D) L-Val n=11	13 5 *	7 3 *		34 14	33 13-23	7 0 *	6 2 *	9 4 *	8 0 *	11 4 *	8 2 *		8 3 *			11 3 *				
	E) L-nLeu n=11	0 0 *	3 0 *	0 0 *	24 2 *	6 2 *	9 0 *	18-29 23	28 22	30 19	12 5 *	0 0 *	10 4 *	4 0 *			4 0 *	22 0 *			
	F) L-Ala n=9	12.5 0 *	22.5 0 *		16 0 *		6.5 0 *	13.5 3 *	19.5 2 *	28 8 *	29-64 26 *	36.5 14 *	22.5 14 *	56.5 33	27.5 5 *			19.5 8 *	0 0 *		
	G) L-Cys n=8		0 0 *	0 0 *	0 0 *					4.5 0 *	0 0 *				20-37 13	22 0	21.5 9.5 *				



* signif. diff. from conditioning stim. *Ital* = not diff. from conditioning stim.

(A) First test, (B) second test. Vertical lines = conditioned stimulus; horizontal lines = behaviorally equal stimuli; diagonal lines = behaviorally similar stimuli; white squares = stimuli behaviorally different from the conditioning stimulus. Bold numbers = range of medians for conditioned stimulus, or median response for other stimuli. Super- and subscript numbers to the right of each square show upper and lower quartiles. *Significantly different from conditioned stimulus response ($P < 0.05$, Wilcoxon rank test, n is given for each conditioning stimulus separately); italics = not significantly different from conditioned stimulus.

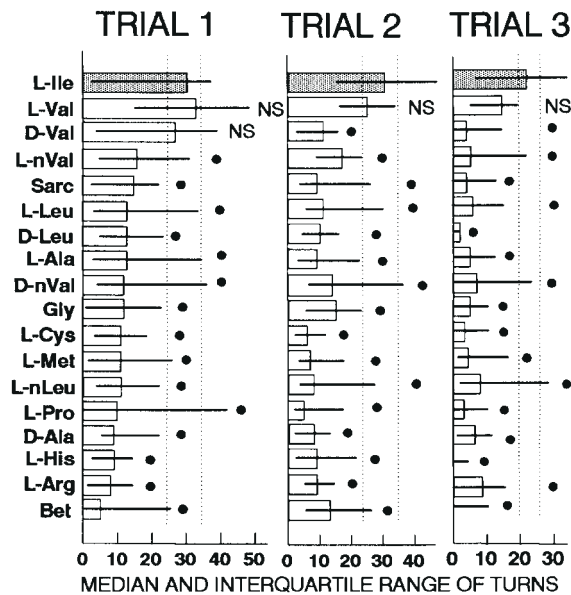


Figure 2 Paradigm 1: responses of L-isoleucine-conditioned (gray bars) brown bullhead catfish to amino acids (white bars) in aquaria without shelters. Horizontal bars and lines indicate the median and interquartile range of turns. The range of medians (nine conditioned sessions for each trial) for the conditioned responses is indicated by the dotted vertical lines. Responses in the second and third trials (**B**, **C**) are arranged by their response magnitude during trial 1 (**A**). Dots indicate a significant ($P < 0.05$) difference between the responses to the conditioned and test stimuli; NS, not significant (Wilcoxon rank test).

Discussion

A greater behavioral response of brown bullhead catfish to the conditioned than to the non-conditioned stimuli made the study of olfactory discrimination and detection of amino acids possible. The basis for this study is the fundamental assumption that animals detect test substances as equal to a conditioned stimulus if the test molecules bind to the same molecular receptors as does the conditioned stimulus, thus exciting the same sensory cells and, after convergence, excite the same glomeruli in the olfactory bulb. A qualitative mismatch between the conditioned and non-conditioned substances at their receptors is most likely to elicit differential activity in the olfactory receptor cells and in the olfactory bulb, thus eliciting different sensations in the brain that are expressed in the current behavioral paradigm as a shorter and less intense food search activity than during stimulation with a conditioned stimulus.

The olfactory sense is required for the discrimination of amino acids in catfish

Although not required for turning and biting/snapping activity—Figure 3A (Valentinčič and Caprio, 1994b), the olfactory organ is as essential for the discrimination of odorants by the brown bullhead catfish (Figure 3B) as it is for the channel catfish (Valentinčič *et al.*, 1994) and goldfish (Zippel *et al.*, 1993). In a previous study, 1-year old channel

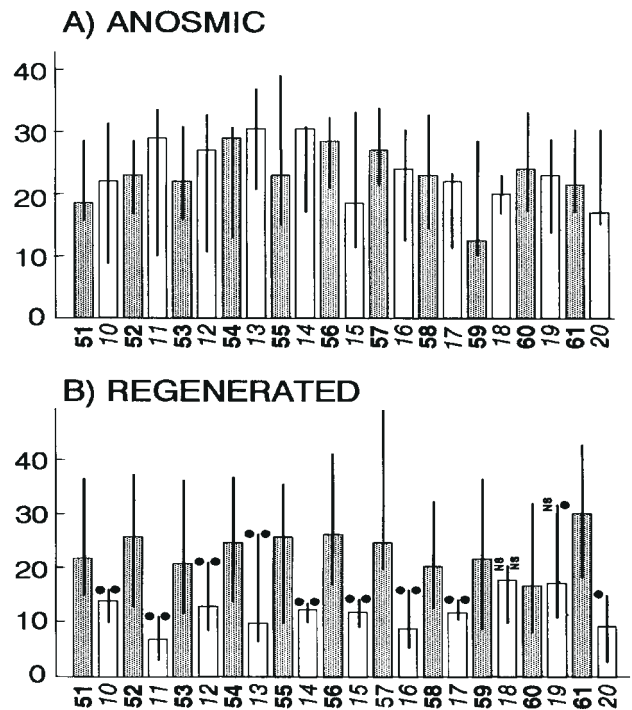


Figure 3 Paradigm 1: responses of anosmic brown bullhead catfish (**A**; $n = 3$) and those with regenerated olfactory lamellae (**B**; $n = 10$). Ordinate, median and interquartile range of turns; abscissa, test number. Vertical bars indicate the median and interquartile range of turns. Solid bars, response to L-alanine in L-alanine-conditioned fish; open bars, responses to the test stimulus, L-arginine hydrochloride. Dots indicate a significant ($P < 0.05$) difference between the responses to the conditioned stimulus, L-alanine, and the test stimulus, L-arginine HCl; NS, not significant.

catfish remained anosmic following bilateral olfactory organ ablation and did not regenerate olfactory lamellae (Valentinčič *et al.*, 1994). The surgical opening of the olfactory organ was regrown by connective tissue and skin yielding permanently anosmic animals (Valentinčič *et al.*, 1994). In contrast, in the present study, with the exception of three brown bullhead catfish which remained anosmic following bilateral olfactory organ ablation, olfactory lamellae of adult brown bullhead catfish partially regenerated, and these animals were capable of being conditioned to single amino acids and of discriminating these odorants from non-conditioned stimuli. Although a 3- to 5-year-old brown bullhead catfish contain ~24 olfactory lamellae in each of its paired olfactory organs, the present results strongly suggest that the receptor neurons contained within a few or even a single lamella are capable of providing sufficient input to the central nervous system concerning odorant quality to allow for odorant discrimination. However, further experiments are needed to clarify the question as to whether brown bullhead catfish with regenerated lamellae are capable of as fine a discrimination of amino acid odorants as intact control fish.

Bullhead versus channel catfish behavior in aquaria with and without shelters

Both channel (Valentinčič and Caprio, 1994a) and bullhead (present report) catfishes in aquaria without shelters and containing black gravel substrate behaved similarly; i.e. both species responded to the conditioned and non-conditioned stimuli with swimming behavior. Critical to the present investigation, however, is that the responses to the conditioned amino acids were approximately two to three times greater than those to the non-conditioned stimuli (Table 1A, B, lines A, C, D). In aquaria with shelters, brown bullhead catfish respond almost exclusively to the conditioned stimulus and to a much lesser extent to non-conditioned stimuli that resemble molecularly the conditioned stimulus. No swimming response occurred to the non-conditioned test stimuli that were not similar molecularly to the conditioned stimulus (Table 1A, B, lines B, E–G). In contrast, channel catfish within shelters were inhibited by the ambient aquarium room lighting and thus did not exit their shelters in response to any amino acid stimulus tested, including the conditioned stimulus (Valentinčič and Caprio, 1994a). Although channel catfish failed to leave the shelter and enter a food search behavior, slight movements of the body and barbels inside the shelters in response to the conditioned stimulus were evident. This photic inhibition of food-searching behavior did not occur in brown bullhead catfish, which have small eyes. The discrimination of olfactory stimuli in aquaria without shelters allows for, both in channel and brown bullhead catfishes, the classification of odorants into (i) conditioned or identical to the conditioned stimulus and (ii) non-conditioned stimuli (i.e. detected by the fish as different from the conditioned stimulus). In aquaria with shelters (tested with brown bullhead catfish only), the stimuli were classified into conditioned (i.e. the fish are as active to the test stimulus as to the conditioned stimulus), non-conditioned (i.e. the fish does not leave its shelter) and stimuli similar to the conditioned stimulus (the fish leaves the shelter and searches, but the response is usually significantly less than observed with the conditioned stimulus). Tested were neutral amino acids with short and long side-chains, basic amino acids and an imino acid.

Evidence for an imino acid receptor (paradigm 1 only)

Brown bullhead catfish detect L-Pro as a unique stimulus, which indicates a narrow tuning of the molecular imino acid receptor(s) for L-Pro. No other L-amino acids in the present report (Table 1A, B, line A) or in a previous study with channel catfish (Valentinčič and Caprio, 1994a) were recognized as equal to L-Pro. The present evidence for an independent receptor site for L-Pro is consistent with results from studies of olfactory responses to binary mixtures of amino acids where a separate and specific L-Pro receptor site was suggested (Caprio and Byrd, 1984). The recent calcium-imaging study of presynaptic olfactory receptor

terminals onto the olfactory bulb in the zebrafish (*Danio rerio*) also clearly indicated a specific localized bulbar pattern of calcium elevation upon stimulation with L-Pro, a pattern distinctly different from those induced by olfactory stimulation with other L-amino acids (Friedrich and Korsching, 1997).

Evidence for basic amino acid receptors (paradigm 2)

In the present experiments, brown bullhead catfish detected the basic amino acids, L-Arg HCl and L-Lys HCl, as similar stimuli. L-Lys HCl was consistently a weaker stimulus than L-Arg HCl (Table 1A, B, line B). Other amino acids were ineffective in releasing swimming activity in L-Arg HCl-conditioned bullhead catfish and were thus detected as different from the conditioning stimulus, L-Arg HCl. These results support the existence of olfactory receptors for basic amino acids previously proposed in channel catfish (Caprio and Byrd, 1984; Caprio *et al.*, 1989; Kang and Caprio, 1991). Existence of a class of basic amino acid receptors different from those to other amino acids was also supported by calcium-imaging experiments of olfactory receptor neuron terminals at the surface of the zebrafish olfactory bulb (Friedrich and Korsching, 1997). Odorant stimulation with L-Arg and L-Lys resulted in a different pattern of olfactory bulb activation from the activity patterns induced by other amino acid odorants. These results suggest both specific olfactory nerve projections from olfactory receptor neurons containing receptors for basic amino acids and a specific chemotopic organization for processing basic amino acid information within the olfactory bulb. That separate receptors exist for basic amino acids different from those for neutral and acidic amino acids does not negate the possibility that independent receptors can also exist in some species for L-Arg and L-Lys, as was previously indicated in the channel catfish (Valentinčič *et al.*, 1994).

Evidence for neutral amino acid receptors

Electrophysiological mixture (Caprio *et al.*, 1989) and cross-adaptation (Caprio and Byrd, 1984) studies of olfactory receptor cells in channel catfish indicated separate receptors for neutral amino acids consisting of long (LCN) and short side-chains (SCN), respectively. The existence of receptors for amino acids within the LCN and SCN categories was also suggested from electrophysiological responses of olfactory receptor neurons to more complex mixtures of neutral amino acids (Kang and Caprio, 1991). These previous results suggest that ictalurid catfish might be capable of discriminating neutral amino acids from each other. The present experiments confirmed this and showed that brown bullhead catfish discriminated at least five different classes of neutral amino acids from each other: (i) L-Val/L-Ile; (ii) L-Ala and neutral amino acids with short side-chains; (iii) L-nLeu/L-nVal/L-Met (neutral amino acids with long and unbranched side-chains); (iv) L-Leu; and (v) L-Cys.

Interestingly, the chemotopic odorant coding proposed recently for zebrafish supports the separate detection of the majority of the previous classes of neutral amino acids (Friedrich and Korsching, 1997).

Discrimination among neutral amino acids with long side-chains

Brown bullhead catfish conditioned to L-Ile (paradigm 1, no shelters) did not discriminate L-Val from L-Ile, and catfish conditioned to L-Val did not discriminate L-Ile from L-Val; however, all other amino acids tested were detected as different from both conditioned stimuli. When in separate experiments this reciprocal inability of brown bullhead catfish to discriminate L-Val from L-Ile was confirmed, both amino acids were tested for impurities. Low concentrations of L-Val (0.28%) in L-Ile and of L-Ile (0.35%) in L-Val provided an additional confirmation that these two amino acids possibly interact with the same receptors. It is noteworthy that L-Leu was always discriminated from other strongly hydrophobic amino acids, including L-Val and L-Ile, indicating that discrimination among LCNs is not based on the hydrophobicity of the compounds, but rather on their specific molecular shapes. These behavioral results in brown bullhead catfish are consistent with the highly similar localized bulbar patterns of calcium increase in the zebrafish in response to L-Val and L-Ile (Friedrich and Korsching, 1997). In addition, this bulbar calcium pattern for L-Val and L-Ile was different from the patterns of calcium increase in response to other amino acids tested, which also corresponds to behavioral evidence that bullhead catfish discriminate L-Val and L-Ile from other neutral amino acids (Figure 2; Table 1A, B, lines C, D).

Discrimination among amino acids with short side-chains (SCNs)

In most cases, L-Ala-conditioned brown bullhead catfish (paradigm 2, shelters) discriminated L-Ala from other SCNs; however, the response to SCNs was always greater than responses to other amino acids tested. Sorting the amino acids tested according to the magnitude of the behavioral response reveals that L-Ala; Gly, L-Ser and β -Ala are located adjacent to each other (Table 1A, B, line F). Neither neutral amino acids with long and branched side-chains nor L-Arg, L-Pro or betaine elicited food search in L-Ala-conditioned brown bullhead catfish, which supports the existence of SCN receptors in the channel catfish (Caprio and Byrd, 1984; Caprio *et al.* 1989). The responsiveness of L-Ala-conditioned brown bullhead catfish in the first test series to other SCNs is indicative of difficult discrimination of similar compounds from L-Ala. With an increased number of conditioning trials, both channel (Valentinčič *et al.*, 1994) and brown bullhead catfish (Table 1 B, line F) started to discriminate L-Ala from other SCNs, indicating an increased saliency of individual amino acid stimuli that with additional training become better discriminated from

the conditioned stimulus. In zebrafish, the surface of the bulb excited by L-Ala is similar to that excited by L-Ser, but the bulbar area excited by Gly is much smaller—see Figure 4 in Friedrich and Korsching (Friedrich and Korsching, 1997).

In an electrophysiological cross-adaptation study, L-Cys only cross-adapted 75% of the response to L-Ala, and L-Ala only cross-adapted 55% of the response to L-Cys, indicating that L-Cys binds to receptors which are at least in part different from those for other SCNs (Caprio and Byrd, 1984). In the present behavioral experiments, with the exception of DL-homocysteine, bullhead catfish conditioned to L-Cys did not respond repeatedly to other SCNs. Thus, DL-homocysteine most likely activates the majority of L-Cys sites, and L-Cys interacts with receptors different from those for L-Ala. Unfortunately for the present comparison, L-Cys was not tested in the zebrafish calcium-imaging study.

In summary, many of our behavioral results in brown bullhead catfishes correlate well with the calcium-imaging data obtained in the zebrafish olfactory bulb (Friedrich and Korsching, 1996), suggesting a similar organization of olfactory receptor sites in these two species and possibly for other teleost fishes comprising the families Ictaluridae and Cyprinidae. The results from both studies suggest relatively independent olfactory receptor(s) across the following different classes of amino acids:

- imino acid (L-Pro);
- basic amino acids (L-Arg and L-Lys);
- neutral amino acids with branched side-chains (L-isoLeu and L-Val);
- neutral amino acids with long, linear side-chains (L-nLeu, L-nVal and L-Met);
- neutral amino acids with long branched side-chain (L-Leu);
- neutral amino acids with short side-chain (L-Ala, L-Ser, Gly and β -Ala);
- amino acids possessing sulfhydryl groups (L-Cys and L-homoCys).

Our behavioral experiments also suggest the likelihood of different, in part overlapping, receptors for some of the individual amino acids comprising a single class of amino acids such as amino acids with short side-chain.

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